STAIN TECHNOLOGY

A Journal devoted to the science of staining; dealin with the nature and uses of biological stains; and touc various related subjects, such as the therapeutic use o bacteriological culture media containing dyes, and micr technic in procedures involving stains.

Editor and business mgr.: H. J. CONN, Geneva, N.

Associate editors: W. C. HOLMES, Washington, D. C.
S. I. KORNHAUSER, Louisville, Ky.
F. B. MALLORY, Boston, Mass.
W. R. TAYLOR, Philadelphia, Pa.

The journal is being issued quarterly at present, pos more often in the future.

The first number appeared in January 1926 and is still a able to those who specify that their subscriptions are to b with January.

Published by the commission on Standardization of Biological Stains.

Subscription for the year: $1.50. For subscriptions ap to the chairman of the Commission: H. J. CONN, Lock t 299, Geneva, N. Y.

Contributions on subjects included in the scope of the journal are welcome. Manuscript should be sent to the editor at the address just given.

STAIN TECHNOLOGY

A Journal devoted to the science of staining; dealing with the nature and uses of biological stains; and touchin various related subjects, such as the therapeutic use of c bacteriological culture media containing dyes, and microsc technic in procedures involving stains.

Editor and business mgr.: H. J. CONN, Geneva, N. Y.

Associate editors: W. C. HOLMES, Washington, D. C.
S. I. KORNHAUSER, Louisville, Ky.
F. B. MALLORY, Boston, Mass.
W. R. TAYLOR, Philadelphia, Pa.

The journal is being issued quarterly at present, poss more often in the future.

The first number appeared in January 1926 and is still av able to those who specify that their subscriptions are to b with January.

Published by the commission on Standardization of Biological Stains.

Subscription for the year: $1.50. For subscriptions ap to the chairman of the Commission: H. J. CONN, Lock 299, Geneva, N. Y.

Contributions on subjects included in the scope of the journal are welcome. Manuscript should be sent to the editor at the address just given.

BIOLOGICAL STAINS

A Handbook on the Nature and Uses of the Dyes Employed in the Biological Laboratory

By H. J. CONN

New York Agricultural Experiment Station

Chairman, Commission on Standardization of Biological Stains

Prepared with the collaboration of

J. A. Ambler, S. I. Kornhauser, F. B. Mallory, and L. W. Sharp

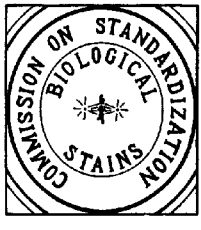
Members of the Executive Committee
of the Commission

SECOND EDITION

Enlarged and revised with the assistance of

W. C. Holmes and R. W. French

and other members of the Executive Committee



Published by the Commission

Geneva, N. Y.

U. S. A.

1929

COMPOSED AND PRINTED BY
W. F. HUMPHREY,
GENEVA, N. Y., U.S.A.

TABLE OF CONTENTS

PREFACE TO SECOND EDITION

IN the four years since the first edition of this book was published so much has been added to our knowledge of stains and staining that a large proportion of what was then written is now out of date. As a result it has been necessary almost to rewrite the book in revising it for the second edition.

At the same time a good deal of new material has been added which should make it more useful. It will be noticed, in particular, that under various dyes, staining formulae and procedures employing these dyes are given in detail. This was not done in the first edition, as it was difficult to decide which procedures to include and which to omit. Obviously it would be impossible to include all staining methods without expanding the book into a text on microscopic technic, an undertaking which did not seem wise. Since publication, however, there has been a demand for such material in the book, and the present edition includes many of the most outstanding staining methods. No claim is made that all important procedures are given. The number outlined in this book has been limited by including only those that have been used in the laboratories of the members of the Commission and are therefore known to give good results with modern American stains. It is felt that their inclusion will make the book a more useful handbook for the laboratory than was the earlier edition.

The members of the Commission Executive Committee who collaborated in the preparation of the first edition were: J. A. Ambler, S. I. Kornhauser, F. B. Mallory, and L. W. Sharp. The members of this committee while the present edition has been in course of preparation, are: R. W. French, S. I. Kornhauser, F. B. Mallory and C. L. Wilson. In addition to the assistance given by these committee members special acknowledgment must be given to Miss Rachel Haynes for assistance in compiling the staining procedures and to Mr. W. C. Holmes, whose criticism of all parts of the manuscript dealing with chemistry has been invaluable.

Acknowledgments are likewise due to the Chemical Foundation who, thanks to the personal interest of their president, Mr. F. P. Garvan, have generously supported the investigations of the Stain Commission; and also to the New York State Agricultural Experiment Station for the laboratory facilities furnished and for making it possible for the chairman to give the necessary time to these investigations and to the preparation of this book.

H. J. CONN, *Chairman,*
Commission on Standardization
of Biological Stains.

Geneva, N. Y., 1929.

PREFACE TO FIRST EDITION

WHEN microscopists first began, in the sixties and seventies, to use stains, the demand for dyes for this purpose was naturally too small to justify a special source of supply. They therefore had to make use of textile dyes, which were then very crude and were not constant in their composition. After a number of years, however, the demand for biological stains grew and a special commercial source of supply for them first appeared in Germany. This was the Grübler Co., later Grübler and Holborn. This company did not manufacture the dyes, as used commonly to be thought in other countries; but on the other hand it cannot be denied that its founder made a distinct contribution to science in making the first effort to secure constancy and reliability in dyes intended solely for the use of the microscopist. It is supposed that he tested dyes under the microscope himself, and if a batch proved satisfactory in his experience bought a supply large enough for a number of years, bottled it under his own label and sold it to biologists. There is no question but that in this way the biologist was furnished with a much more reliable line of stains than if he had been obliged to buy directly from the dye manufacturers; but it was an empirical method of standardization and there was nothing to prevent different batches of some dye secured by this company from varying considerably in their composition. Such upon investigation has proved to be the case.

Altho a great service was done to biologists by this company in the latter part of the nineteenth century, such methods of standardization are not in keeping with modern scientific knowledge. A recent cooperative undertaking has therefore been organized in America to put the standardization of stains upon a scientific basis. This undertaking started after the war had caused a shortage of stains, with the object of securing a reliable supply when the foreign sources were unavailable. It has since then been widened in its scope; and now that the foreign products are again available, the purpose of the work is to effect a scientific standardization of stains whether derived from foreign or domestic sources. As a matter of fact, so far only domestic samples have been considered. This has not been because of any prejudice against foreign stains, but because of practical difficulties; it is, in brief, difficult to test each batch before it is put on the market when the concern handling it is in Europe.

The organization thru which this work is being carried on is known as the Commission on Standardization of Biological Stains. It was organized in 1922 under the auspices of the National Research Council and is still affiliated with it, altho now no longer a

part of the larger body. It is in effect a coordinating committee representing the American Chemical Society, the American Society of Bacteriologists, the Society of American Zoologists, the Botanical Society of America, the American Association of Pathologists and Bacteriologists and the American Association of Anatomists. It has a membership of about sixty biologists, members of the various societies just mentioned, who assist in the examination and testing of stains, each in those particular lines of technic with which he is especially familiar. It has secured the cooperation of chemists, dye manufacturers and stain dealers, so as to be sure that the needs of biologists can be immediately reflected in the supply of stains on the market. Its affairs are managed by an executive committee of five members, the present members of which represent bacteriology, botany, dye chemistry, pathology, and zoology, respectively. This executive committee has undertaken the preparation of this book. The authorship of the book has been assumed by the chairman of the committee, however, in order to fix the responsibility and to make bibliographic references to it simpler than in the case of plural authorship; but the assistance of the other committee members in the work has been so great that they may be practically considered co-authors of the book. The chairman of the committee, therefore, wishes to take this occasion to acknowledge the invaluable assistance given by these other members. Without their cooperation such an undertaking would have been impossible.

The chief object of the book is to present in logical form the information which has been accumulating in the hands of the Commission since it was organized. It is neither a treatise on dye chemistry nor one on microscopy; altho it contains information in both fields. It is an effort to present in a form acceptable to biologists the principles of dye chemistry so far as they have a bearing on biological stains; and to discuss the suitability of the different dyes for various biological purposes, presenting data partly original and partly drawn from the literature. The subject matter is realized to be incomplete, particularly that part of it which deals with the biological uses of dyes. An effort has been made to list the most important present uses of stains, and of the obsolete uses to mention those of historical significance; but it is realized that there must be many omissions. It is hoped that readers of the book will cooperate by calling to the author's attention places where the treatment of any subject seems inadequate.

H. J. Conn, *Chairman,*
Commission on Standardization
of Biological Stains.

Geneva, N. Y., 1925.

CHAPTER I

HISTORY OF STAINING

CONSIDERING how dependent microscopists are today upon the use of stains, it is hard to realize that much important work had been done with the microscope before the use of stains was attempted. Altho natural dyes such as carmin and indigo were well known in the early days of the microscope, their use in staining microscopic preparations does not seem to have become common till about 1850; and anilin dyes were not put on the market until 1856. Yet anyone who has studied the history of biology must realize that many discoveries had been made with the microscope before this period.

It is safe to say, nevertheless, that the use of stains revolutionized microscopic technic. The early microscopists were able to make much progress without stains because of their painstaking diligence. The work without stains must have been extremely difficult, and it is hard on reading some of the old publications to believe that some of the minute structures described were actually seen. Few users of the microscope today would be likely to have either the patience or the eyesight to do the work described in those early days. The fact that the microscope is now being used successfully in the hands of so many students who would not think of comparing themselves with the pioneers in microscopy is due to the use of stains more than to any other factor—altho of course no one can deny that modern improvements in the microscope have also played a part of great importance.

The first use of a dye in microscopic work seems to have been by Hill (1770)* who a century and a half ago employed carmin in studying the microscopic structure of timber. Hill was much ahead of his time, and there is no further reference to a use of this dye in microscopy till Ehrenberg (1838) used powdered indigo and carmin in the study of living microscopic organisms. Assuming that the dye particles would be consumed bodily, he thought he could trace the digestive system by the location of the dye in the body. Certain colored zones or bands were thus revealed; and regarding each of these colored spots as a stomach, Ehrenberg named the group of organisms "Polygastrica." Inasmuch as the group included bacteria and protozoa, the name was somewhat of a misnomer. It is interesting, however, to realize that Ehrenberg's technic is still used to demonstrate the ability of protozoa to engulf food particles.

The early history of staining from this time on is given in a very interesting manner by Gierke (1888–5) and by Mann (1902), one

*For references cited see Bibliography pp. 204–216.

or the other of which sources is recommended to anyone desiring a more detailed account than is given here.

Besides the fairly common application of iodine to microscopic preparations there seems to have been no further effort to color objects under the microscope until the middle of the century. At this time carmin was used almost simultaneously by two botanists, Göppert and Cohn (1849) and by a zoologist Corti (1851). Göppert and Cohn used the dye to assist them in studying the rotation of the cell contents of *Nitella flexilis*. Corti's work is overlooked by both Gierke and by Mann; but he states definitely (p. 143) that to observe distinctly the epithelial cells one should color them lightly with a solution of sugar or of carmin in a mixture of half water and half alcohol. This work was very promptly followed by another use of the same dye—the staining of chlorophyl granules in plants—by Hartig (1854 and 1858). In studying the nucleus of cells he made use not only of carmin but of litmus and black ink; and he observed that while albumin and gelatin were easily stained, the dyes had no action on such material as gums and mucin. Altho these authors did careful scientific work and certainly were the first users of dyes for histological purposes, their work apparently attracted no attention at the time. The real introduction of biological stains was made by Gerlach (1858).

Altho Gerlach did not discover the action of dyes on microscopic objects, nor was he the first to use carmin, nevertheless he should be, and generally is, considered the father of the technic of staining. Having observed that tissues became colored after injection with a poorly prepared carmin gelatin, he devised the scheme of preparing an ammoniacal carmin. He was therefore the first to use ammonium carminate, which is so nearly indispensible to modern histologists. His early efforts with it, however, were unsuccessful—until he had a lucky accident which revealed the source of his trouble and opened up the way for further work. He happened to leave a section of nervous tissue, which had been hardened in potassium bichromate, over night in a very dilute carmin solution. When he examined it twenty-four hours later he found that it was beautifully stained, with fine differentiation of nerve fibers and nerve cells. His earlier failures had been due to the use of too strong a solution of the dye. This gave the key which helped solve the problem of tissue staining. Advances came quickly after that; for Gerlach had shown the way, and others merely had to follow.

These advances were not wholly with carmin; altho at first the anilin dyes were scarcely known and the number of stains available to microscopists was quite limited. Indigo was first employed by Maschke (1859) who was familiar with Hartig's work but not with Gerlach's. It is stated (anonymous, 1865) that Thiersch and Müller had just developed a technic employing carminates in combination with oxalic acid, and a year later Schweigger-Seidel and

Dogiel (1866) introduced a combination of carminates with acetic acid. Hematoxylin was first introduced as an histological stain by Böhmer (1865), altho a previous rather unscientific attempt had been made by Waldeyer (1864) to stain axis-cylinders by means of the watery extract of logwood. Böhmer's greater success was due to his use of haematoxylin crystals in combination with alum, either by accident, or else because he knew that it was frequently used as a mordant in textile dyeing. Slightly later Frey (1868) showed that similar results could be obtained by mixing the mordant with the solution in which the tissues were fixed before they were stained.

Double staining was introduced at about this same period, when Schwarz (1867) proposed fixing tissue in creosote and acetic acid, then staining 24 hours in very dilute ammonium carminate, and subsequently washing and staining for two hours in picric acid. A year later Ranvier (1868) first used a picro-carmin stain to obtain the same results by a single procedure.

Anilin dyes had become commercial articles before all these advances with the natural dyes had been made, the first one having been introduced in 1856 when Perkin prepared mauveine. Fuchsin, under the name of anilin red, appeared in 1858. The first suggestion of their use in histology seems to have been made by Beneke (1862), who used acetic acid colored with a lilac anilin, probably a mauveine or anilin violet; while two years later Waldeyer (1864) used anilin red (fuchsin) and also a blue and a violet anilin dye. The latter investigator observed the ability of fuchsin to stain nuclei more deeply than cytoplasm and the axis cylinder more deeply than the medulary sheath of nerves.

The principle of differentiation following staining was soon introduced. Böttcher (1869) differentiated his sections by partially decolorizing with alcohol after staining with rosanilin nitrate. A very similar method was later published by Hermann (1875), who is often mistakenly given the credit for originating the principle. Later the same procedure was further investigated by Flemming (1881) who tried both acid and basic dyes, finding that the method was satisfactory only in the case of the latter group.

Gierke (l. c.) in his historical discussion of staining says that the history up to his day (1884) was divided into three periods, each occupying a decade. The first decade, the fifties, was characterized by a few important but unrelated discoveries, which ended in the work of Gerlach, each investigator following up accidental observations on the staining powers of carmin and the other well-known dyes of those days. After Gerlach's work the development of the technic in the sixties was more rapid and depended less upon chance success by the individual investigator; the effort was made to use similarly all the dyes and metallic colors then available. The next decade would have had much less left to develop in this line if it had not been that by this time the great variety of anilin dyes

were available and microscopists were constantly finding new uses for them. Gierke wondered if there would be any opportunity for equal development during the ten years to follow his paper.

That development did not stop in his day is well known. Scarcely a year has passed without the introduction of some new staining technic of considerable importance. Sometimes dyes hitherto unknown to the biologist have been shown to be valuable in bringing out some particular structure; at other times new combinations of dyes have proved of special value for other purposes; while by other investigators it has been shown that old methods, used with modern refinements of apparatus and technic, may bring out details not dreamed of by the early histologists. But the farther this work has progressed the more the microscopist has become dependent upon his supply houses to furnish him reliable stains, so carefully purchased or manufactured that each lot ordered could be counted upon to duplicate the last.

The preface of this book describes briefly how a company was formed in Germany to meet the demand for dyes for staining purposes that developed during the last three decades of the nineteenth century, and how the recent post-war conditions, together with the modern demand for a more scientific basis for the industry, led to the establishment in America of a Commission on Standardization of Biological Stains. The work of the Commission is two-fold. First, by cooperation of various biologists and chemists it is planning to get together all the available information concerning the nature of dyes as related to their use for various purposes in microscopic technic; secondly, by working with the manufacturers it is trying to see that the supply of stains available in America is of the highest possible quality, as judged by their performance in actual laboratory use.

The first of these purposes has inspired this book, which is now in its second edition, and at the same time has led to the publication by the Commission of a small journal, Stain Technology. The second object is being brought about by the plan of certifying stains.

MODERN STANDARDIZATION OF STAINS

The extreme difficulty of drawing up any chemical or physical standards for a satisfactory stain has made it necessary to adopt a rather empirical method of standardization. The specifications for the various stains drawn up by the Commission and published in the past contain,* in addition to the chemical and physical requirements, a clause essentially as follows: "The sample must prove satisfactory in the.....method for staining..... This point should be tested by someone familiar with the technic in question." It is hoped that eventually it may be discovered just

*See first edition of this book pp. 132–7. Also Stain Tech. 2, 27–30, 1927.

what physico-chemical characteristics indicate a satisfactory stain in each particular instance and that then such clauses as these may be dropped from the specifications; but that time is still some distance in the future.

In order to apply such a requirement as that quoted above—especially in view of the fact that two batches of a dye may differ in their staining qualities without detectable difference in chemical characteristics—it is necessary to submit each individual batch of any stain to an expert in the procedures for which it is used. This is done with all the stains sent in to the Commission for certification, as well as having them submitted to a physico-chemical analysis.

The certification label on any bottle of stain means, therefore, five things: (1) a sample of the batch bearing the label has been submitted to the Commission for testing and a portion of the sample is permanently on file in the chairman's office; (2) the sample proves true to type, as judged by spectrophotometric tests; (3) its dye content is up to specification and is correctly indicated on the label; (4) it has been tested by experts in the procedures named on the label and has been found satisfactory by them; and lastly, (5) no other batch can be sold under the same certification number except by such a flagrant breach of confidence on the part of the company as to risk losing the good will of the Commission.

Two forms of labels have been used by the Commission for this purpose: one a large label 1½ by 3½ inches, on which the name of the stain is printed either with or without the name of the manufacturer, the other much smaller, 1 by 1¾ inches, bearing nothing but the certification statement. This certification statement, appearing on both forms of labels is as follows:

"Found satisfactory by Commission on Standardization of Biological Stains for purposes mentioned on label. Use for other purposes not contra-indicated unless specifically so stated on said label. Will users please report any unsatisfactory results to the chairman, H. J. Conn, Geneva, N. Y."

These labels are printed in dark blue ink on a paper bearing the Commission seal (see title page of this book) as a continuous background design similar to that on safety check paper.

The first certified stain, methylene blue, was put on the market in the summer of 1923. Before the end of 1923 one other dye, safranin, was added to the list, and during 1924 eight more, thus making ten certified stains in all. By the end of 1925 the list had increased to twenty-nine and by the end of 1926 to thirty-seven. By that time nearly all the more commonly used stains were on the certification basis and the subsequent increase has, therefore, been slower. At the present time the total number of dyes certified is forty-three. A list of them is given on page 197.

Not all the samples submitted for certification have been satisfactory, and thruout the six years a certain proportion of the samples have been rejected. The total number of samples submitted up to the end of 1928 has been 250 and of these 40, or 16%, have been rejected. A study of the number of samples rejected each year indicates a distinct improvement in the stain supply during the period. In 1923 the rejected samples constituted 45% of the total submitted; in 1924, 30%; in 1925, 13%; in 1926, 7%; in 1927, 12%; while it was only 5% in 1928.

GOVERNMENT SPECIFICATIONS

It was pointed out in Stain Technology a few years ago (French 1926a) that government departments have a practical problem involved in assigning specifications for stains which is quite different from that which the Stain Commission has been trying to meet.

Two conferences have been held within the last year which seem likely to harmonize the two points of view and secure close coöperation in the matter in the future.

The specifications drawn up by the Stain Commission have merely been intended to secure a product that would probably work satisfactorily, and upon which the Commission certification label could be placed if the actual tests of the sample were favorable. From the standpoint of the purchaser the main emphasis has been upon the certification label of the Commission, not on the published specifications.

In the case of the government purchasing agencies, as pointed out by French, the procedure has to be entirely different. The government, as a purchaser, has to place its reliance upon specifications and can not specify a stain certified by the Commission. As a matter of fact any company trying to sell a stain to the government can claim that their product, altho not certified, is just as good as the certified stain, and the government may have great difficulty in proving the contrary. For this reason it has been evident that the government should draw up specifications for stains which would assure a good product without excluding any stain that would be satisfactory in actual use. The Commission's specifications, as pointed out by French, have never been adequate for this purpose.

To secure specifications of the type necessary for the government has meant considerable work. The Stain Commission would gladly have published such specifications in the first place had the necessary information been available. Since this information was not at hand, the certification method of securing reliable samples was adopted. At present, however, the Army Medical Museum, in coöperation with the Color Laboratory of the Department of Agriculture, has been obtaining the necessary data in connection with several stains. This information is being placed at the disposal of a committee of the Army, who plan to submit the specifi-

cations when they are prepared to the Surgeon General's office for adoption by the Medical Department. It is quite possible that these stain specifications will be adopted subsequently by the Federal Specifications Board. These specifications are being drawn up in close coöperation with the Biological Stain Commission.

It is planned to determine in the future whether samples submitted for certification meet these specifications. If a sample does not meet them it will not necessarily be refused certification provided it proves satisfactory in actual use. Arrangements will be made, however, to furnish the proper government agencies with the information as to whether or not the sample does meet their specifications and they will thus have at their disposal information to employ in case a sample of any particular certification number is submitted to one of their purchasing agencies.

CHAPTER II

THE GENERAL NATURE OF DYES AND THEIR CLASSIFICATION

DYES are generally classed in two groups, the natural and the artificial. The former class is now of relatively smaller importance from the standpoint of the manufacturer and the textile dyer; for the artificial dyes far outnumber them and the advancement of science is gradually making it possible to produce many of the formerly natural dyes by artificial means. It just happens that one or two natural dyes, the derivatives of cochineal and logwood extract (see Chap. IX) are among the most valuable biological stains; but the natural dyes in general are so few in number that they can be practically disregarded in considering the general chemical nature of dyes.

Because the first artificial dyes were produced from anilin, all of this class are often called "anilin dyes," altho there are now a large number of them which bear no relation to this compound and are not derived from it. Therefore the term is now quite largely being replaced by the more correct expression "coal-tar dyes," since all of them are made by chemical transformations from one or more substances found in coal-tar.

Comparatively little is known concerning the chemistry of the natural dyes; but the synthetic dyes have been carefully studied and volumes have been written concerning their chemical composition and its relation to their behavior. Much of this may well be ignored by the biologist who employs dyes merely for staining microscopic objects. A certain familiarity with the general principles is useful, however, in helping him to employ stains scientifically. This is particularly true now that there is a growing tendency to use dyes in biological work as microchemical reagents. The brief discussion that follows is intended to help the biologist to understand merely the fundamental principles of dye chemistry.

BENZENE

All coal-tar dyes may be considered as derivatives of the hydrocarbon, benzene, C_6H_6, which is the mother substance of the very important aromatic series of organic compounds. It is an unusual chemical compound in many respects, and it will be well, in order to understand the structure of dyestuffs, to review briefly one theory of its structure which accounts for many of its properties. The molecule of benzene is composed of six carbon atoms combined with six hydrogen atoms in such a way that each hydrogen atom is identical in all its reactions with every other hydrogen atom in the molecule. Now a carbon atom is considered to have in all cases four valency bonds, that is it is capable of uniting chemically with

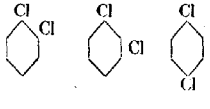
four atoms of hydrogen which has a valency of one. The simplest and best way of expressing these facts by a structural formula is shown in the figure:

The double bonds in this ring cannot be considered as stationary; for, if they were, a compound in which two adjacent hydrogen atoms had been replaced by other elements or radicals should occur in two different forms according to whether there were a single or a double bond between the two carbon atoms to which the substituting elements or groups were attached—which never proves to be, the case. Hence the double bonds must be considered as mobile, each pair continually oscillating back and forth between the carbon atom bearing it and the two adjoining carbon atoms.

In practice the formula for benzene is abbreviated to a simple hexagon:

in which each corner represents a carbon atom. If no chemical symbol is placed outside the ring at any corner, it is understood that an atom of hydrogen is attached at that point. This configuration is spoken of as the "benzene ring." When the symbol of some element or radical is written at a corner, it means that the hydrogen atom at that point has been replaced by the element or radical to which the symbol refers.

When two hydrogen atoms are replaced there are only three possible positions in the molecule which the replacing groups, or substituents, can take, as shown by the following figures, using chlorine as the substituent:

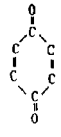


In the first formula, the substituents are said to be in the "ortho" position to each other; in the second they are in the "meta" position, and in the third in the "para" position. These three compounds are called respectively: ortho-dichlorobenzene, meta-dichlorobenzene, and para-dichlorobenzene. The three prefixes are commonly shortened to the respective initials: "o-" "m-," and

"p-." When the compound is complex it is customary to number the corners of the ring thus:

In naming a compound in this way, the number of the corner to which a group is attached is given immediately before the name of the group. Thus, the three compounds shown above may be called respectively: 1, 2-dichlorobenzene, 1, 3-dichlorobenzene and 1, 4-dichlorobenzene.

There is another type of substitution in the benzene ring which is very important in dye chemistry. Two atoms or groups having two valency bonds instead of one may also replace two hydrogen atoms, provided the replacement takes place simultaneously and the hydrogen atoms replaced are situated either in the ortho or in the para position to each other. Thus two oxygen atoms (which are bivalent) may replace two hydrogen atoms (which are monovalent) forming the compound known as quinone $C_6H_4O_2$, the formula for which is

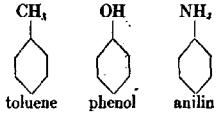


or as commonly written

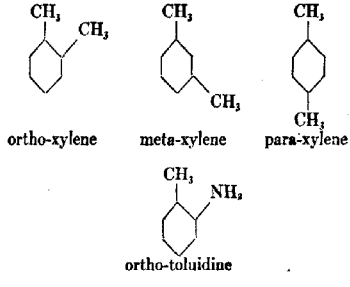
In printed formulae, such as those that follow in this book, the quinone ring is often abbreviated still further by omitting the double bonds within the ring. The substituent atoms or groups may or may not be alike, so long as both have two valency bonds entering into the combination. This type of substitution involves a rearranging of the double valency bonds in the benzene ring; and in compounds of this type, called quinoid compounds, the double bonds are supposed to be fixed, not mobile as in benzene. This change of the valency bonds takes place very readily in many dyes, and certain peculiarities of their behavior are explained by it; (see for example p. 134).

Three mono-substitution products of benzene are of importance in considering the structure of dyes, namely; toluene or methyl-

benzene, $C_6H_5 \cdot CH_3$; phenol, carbolic acid or phenylic acid, $C_6H_5 \cdot OH$; and anilin or phenyl amine, $C_6H_5 \cdot NH_2$. Their constitutional formulae are as follows:



Two important di-substitution products are xylene or dimethyl benzene $C_6H_4 \cdot (CH_3)_2$, and toluidine, $C_6H_4 \cdot CH_3 \cdot NH_2$. Both of these occur in the above mentioned three isomeric forms, as shown below for xylene:

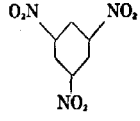


CHROMOPHORES, CHROMOGENS, AND AUXOCHROMES

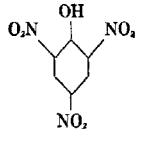
Certain groups of elements are known as chromophores because when they occur in a benzene derivative they impart to the compound the property of color. The benzene compounds containing chromophore radicals are known as chromogens. A chromogen, however, altho it is colored, is not a dye, in that it possesses no affinity for fibers or tissues. It may coat them, but only mechanically, and it will be easily removed by mechanical processes. That is, it will not "take." (See, however, the discussion of fat stains, p. 42). In order for a substance to be a dye, it must contain in addition to the chromophore group, a group which imparts to the compound the property of electrolytic dissociation. Such auxiliary groups are known as auxochromes. They may slightly alter the shade of the dye, but are not the cause of the color. Their function is to furnish salt-forming properties to the compound. Certain chromophoric groups have also slight auxochromatic properties.

To illustrate these different types of groups, let us take a typical example. The nitro group ($-NO_2$) is a chromophore. When three

of these groups displace three hydrogen atoms in a benzene molecule, we have the compound trinitrobenzene,



which is yellow. It is not a dye, however, but is a chromogen. It is insoluble in water, and is neither an acid nor a base; that is, it does not dissociate electrolytically and consequently cannot form salts with either alkalies or acids. If, however, one more hydrogen atom is replaced, this time with the hydroxyl group (-OH), which is an auxochrome, the resulting compound,

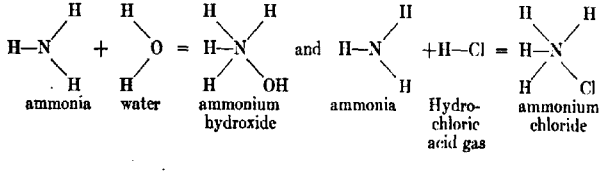


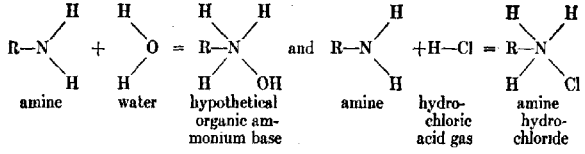
is an acid, capable of electrolytic dissociation and of forming salts with alkalies. It is the familiar substance picric acid, and is a yellow dye.

It will thus be seen that the color of picric acid is due to the chromophoric nitro groups, and that its dyeing properties are due to the auxochromic hydroxyl group. If the nitro groups be reduced to amino groups (-NH_2), which are not chromophores, the resulting compound is colorless and hence is not a dye.

Summing up, we arrive at the definition of a dye as an organic compound which contains chromophoric and auxochromic groups attached to benzene rings, the color being attributable to the chromophores and the dyeing property to the salt-forming auxochromes.

Some auxochromes are basic, e.g., the amino group (-NH_2), while others are acidic, e.g., the hydroxyl group (-OH). The amino group owes its basic character (which it transmits to the whole molecule) to the ability of its nitrogen atom to become pentavalent by the addition of the elements of water (or of an acid), just as in the case of ammonia; thus:





The hydroxyl group, on the other hand, is weakly acidic, as it can furnish hydrogen ions by electrolytic dissociation. The more of either one of these two groups in a compound, the stronger base or acid it becomes. If there is one of each, the basic character of the amino group predominates, but is weakened by the influence of the acidic hydroxyl group. The strength of both groups is also influenced by other groups or atoms in the compound; thus, for example, the chromophore $-NO_2$, altho incapable in itself of conferring acid properties to the compound, exerts an influence to make any hydroxyl group in the compound more strongly acidic, in other words to become more highly dissociated electrolytically.

One other group of atoms encountered in dye chemistry needs explanation, namely the sulfonic group, $-SO_3H$. It is a salt-forming group of strongly acidic character, in that it suffers extensive electrolytic dissociation. This group, however, is only very feebly auxochromic. Its function is to render a dye soluble in water, or to change an otherwise basic dye into an acidic one, as in the case of the fuchsins, where the strongly basic "fuchsins" are changed into the strongly acid "acid fuchsins" merely by the introduction of sulfonic groups into the former. A compound which contains a chromophore group and a sulfonic group is not a dye, however, unless there is also present a true auxochrome group.

From what has been said above, it is not to be presumed that the dyes of commerce are actually bases or acids. Generally the basic dyes are sold as salts of a colorless acid, such as hydrochloric, sulfuric, oxalic or acetic acid. Likewise the acid dyes are sold as their sodium, potassium, calcium or ammonium salts. Occasionally the basic dyes are sold as the free bases, as for example the oil soluble dyes see p. 49). When a basic dye which is ordinarily sold in the form of a salt comes into commerce as the free base, it is customary to use the word "base" immediately after the name of the dye. Thus, "basic fuschin" indicates a salt of fuchsin with a colorless acid, while "fuchsin, base" indicates fuchsin itself, not combined with an acid.

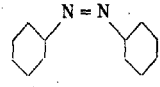
THE CHROMOPHORES

As stated above, every dye contains at least one group of atoms known as a chromophore, which is regarded as being responsible for the colored properties of the compounds in which it occurs. Some of these chromophores have a basic character, others acid. There are only a comparatively small number of them which enter

into the usual biological stains, and only these need be considered here. They are as follows:

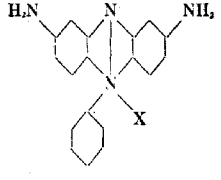
BASIC CHROMOPHORES

1. *The azo group*, —N=N—, which is found in all azo dyes, of which methyl orange and Bismarck brown are well known examples. In all these dyes, a benzene ring is attached to each nitrogen atom. All the dyes of this group may be looked upon as derivatives of azobenzene,

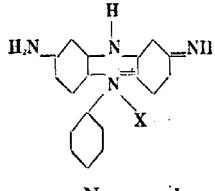


2. *The azin group*,

which is found in phenazins, of which neutral red and the safranins are good representatives. The skeleton formula of a safranin is:

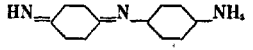


in which x represents the negative ion of a monobasic acid such as hydrochloric, acetic, nitric or sulfuric. This chromophore is capable of variety of rearrangements of its valency bonds, as the bond between the two nitrogen atoms may disappear and the compound assume a quinoid structure, as for example the following grouping:

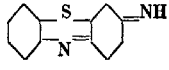


3. *The indamin group*, —N=, as observed in the indamins, thiazins, and so forth. Methylene blue is the best known representative of this group. In these dyes, two benzene rings are at-

tached to the nitrogen atom, one of these being in the quinoid form and hence adding a second chromophore. The typical indamin formula is:



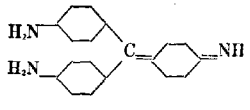
In the thiazins, such as methylene blue, the two benzene rings are further joined together by a sulfur atom, forming three closed rings of atoms. The simplest thiazin base would be:



ACID CHROMOPHORES

1. *The nitro group,* $-NO_2$, as in picric acid
2. *The quinoid benzene ring,*

which occurs in a long series of dyes, such as the indamins above mentioned, the xanthenes and the di- and tri-phenyl methanes, which include many well known stains, such as rosolic acid, fuchsin, methyl green and the methyl violets. A typical triphenyl methane formula is that of pararosanilin, base:

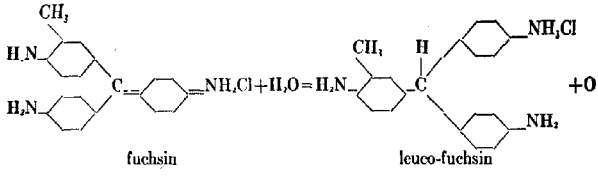


LEUCO COMPOUNDS

The different chromophores differ considerably from one another, but they all have one property in common. In the language of chemistry, they all have unsatisfied affinities for hydrogen; or in other words, they are all easily reduceable, for combining with hydrogen is the opposite of oxidation and is, therefore, reduction. The nitro group may be reduced to an amino group; in the azin group the bond between the nitrogen atoms may break and two hydrogen atoms be taken on; while in the various chromophores with double bonds (such as the quinoid ring) the double bond may break and hydrogen atoms become attached to the valences thus freed.

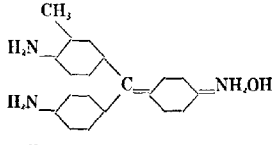
Now in every case this reduction destroys the chromophore group, and as a result the compound loses its color. In other words a dye retains its color only as long as its affinities for hydrogen are

not completely satisfied. These colorless compounds are known as leuco compounds; thus fuchsin yields leuco-fuchsin on reduction, and methylene blue reduces to leuco-methylene-blue. For example:

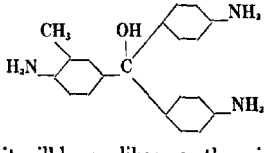


Ordinarily this reaction is reversable under conditions favoring oxidation. It is of especial significance to the bacteriologist, as dyes can often be used as indicators of reduction.

Certain dyes form a still different type of leuco compound, often called a "leuco-base." We have seen that the basic dyes ordinarily occur as salts of some colorless acid; now, in the case of certain dyes, notably the tri-phenyl methanes and xanthenes (Chapters VI and VII), as soon as the acid radical is removed, the compound becomes colorless. This is because a rearrangement of the atoms in the molecule takes place upon neutralization so as to give, not the true dye base, but a compound known as a carbinol (see p. 86) in which the chromophore does not occur. Thus the theoretical base of fuchsin which should be obtained upon removal of the acid radical is:



The compound actually formed, however, is the pseudo-base or carbinol:



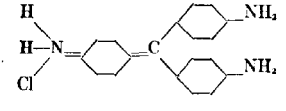
In this compound, it will be readily seen, there is no chromophore; hence it is colorless. These pseudo-bases are of little significance to the biologist, but they are of importance to the dye manufacturer as intermediates in the preparation of dyes.

In the case of many acid dyes the chromophore is similarly broken by a rearrangement of the atoms which occurs on neutrali-

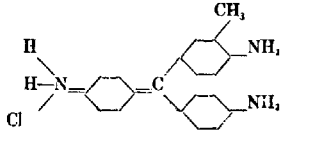
zation. This reaction is ordinarily very readily reversable and makes such dyes useful indicators of acidity. It is discussed more fully under acid fuchsin (p. 98) and phenolphthalein (p. 134).

CLASSIFICATION OF DYES

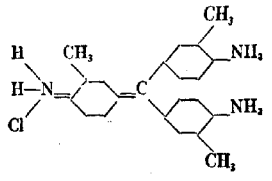
On the basis of the chromophore present the simple synthetic dyes are classified into several groups. If each of these groups were characterized by a single color or by a few closely related colors, dye chemistry would be a comparatively simple proposition. As a matter of fact a single chromophore may occur in dyes of practically all colors of the rainbow. It is ordinarily impossible to determine, *a priori*, from the chemical formula of a dye what particular color the compound may have; but there is, nevertheless, a certain general rule which correlates chemical formula with color. In any group of compounds, the simpler ones are converted into the more complex by substitution of radicals for hydrogen atoms. In the dyes the substituents are generally methyl or ethyl groups, or sometimes phenyl groups. Now the general rule is that the larger the number of hydrogen atoms that have been replaced by these groups the deeper the color. The tendency is for the color of the simplest dyes in any group of homologous compounds to be yellow, passing thru red to violets and then greens and blues, as the homologs become higher thru the introduction of successively larger numbers of methyl or other substituting groups. Thus the compound pararosanilin, which is very frequently sold as basic fuchsin, but should more properly be called basic rubin, is a triphenyl methane, with an amino group attached to each benzene ring, but without any methyl groups; thus:



Rosanilin, which is similar in composition, but contains one methyl group attached to one of the benzene rings,

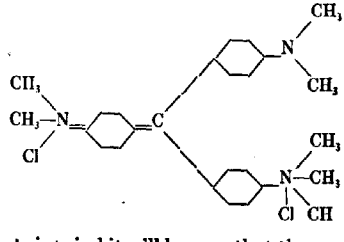


is a red very similar to pararosanilin but with less of a yellowish cast. Now another methyl group may be introduced into each of the other two benzene rings, and each one successively deepens the shade of red, so that the highest homolog of the series, new fuchsin:



has a more bluish cast than any of the others. Thus basic fuchsins can vary considerably in their shade according to the proportions in which these four possible components may be mixed.

It is also possible in another way to deepen the color of pararosanilin still further, namely by introducing methyl groups into the amino radicals instead of directly on the benzene rings. Thus the methyl violets are obtained; and the more methyl groups introduced the bluer the violet, until when all six available hydrogen atoms are thus substituted, crystal violet, the deepest of them all, is obtained. By using three ethyl groups instead of methyl, Hoffman violet or dahlia is formed, which is deeper in color than the trimethyl compound, due to the heavier groups introduced. If three phenyl groups (i.e., the benzene ring (C_6H_5-) are introduced instead of methyl or ethyl, the color is still further deepened, the resulting dye being spirit blue. Further, it is possible to introduce another methyl group into crystal violet, by addition of methyl iodide (or chloride) to one of the trivalent nitrogen atoms, whereby its valency is increased to five, and a green dye, methyl green, is produced:



With these facts in mind it will be seen that the grouping of dyes as based upon these chromophores does not classify them in relation to their color. It is a useful classification, however, because it puts together those that have similar chemical structure. The important biological dyes, thus classified, fall into the following groups:

1. The nitro dyes.
   e.g., *picric acid.*
2. The azo group.
   e.g., *methyl orange, Bismark brown, orange G, congo red, Sudan III and Sudan IV.*

3. The oxyquinone group.
   e.g., *alizarin*
4. The quinone-imide group, including
   (a) Indamins
   (b) Thiazins; e.g., *thionin, toluidine blue, methylene blue,*
   (c) Oxazins; e.g., *brilliant cresyl blue, Nile blue.*
   (d) Azins, including
      (i) Amido-azins; e.g., *neutral red.*
      (ii) Safranins; e.g., *safranin O, magdala red.*
      (iii) Indulins; e.g., *nigrosin.*
5. The phenyl-methane dyes, including
   (a) diphenyl-methanes, e.g., *auramin.*
   (b) Diamino tri-phenyl methanes; e.g., *malachite green, brilliant green, light green.*
   (c) Triamino tri-phenyl methanes; e.g., *basic fuchsin, acid fuchsin, methyl violet, gentian violet, methyl green, anilin blue.*
   (d) Hydroxy tri-phenyl methanes (Rosolic acids); e.g., *aurin, corallin red.*
6. The xanthene dyes, including
   (a) Pyronins; e.g., *pyronin G* and *B.*
   (b) Rhodamines; e.g., *Rhodamine B.*
   (c) Fluorane derivatives; e.g., *eosins, erythrosin, rose bengal.*
   (d) Phenolphthalein and the sulphonphthaleins.

DYE NOMENCLATURE

Very little system has been used in naming dyes, and as a result their nomenclature is extremely confused. Generally the manufacturer of a dye which he thinks is new or which he wishes the public to consider a new dye sells it under a new name which is not intended to give any clue as to the nature of the dye. If the manufacturer knows that the name is a mere synonym of one already in use he does not say so, for he wishes to encourage the sale of his own product rather than that of some other dye maker. Accordingly it has been left for others, who are not financially interested, to work out the synonymy of the dyes; and the list of names that are found to apply to a single dye is sometimes amazing.

With the dyes in general so unsystematically named, it is natural that the same confusion should reign in the nomenclature of biological stains. This confusion is very unfortunate, for it often misleads the biologist as to just what he is doing. For example, some histologist may have on hand a bottle of stain labeled dahlia and he may find it useful for some new technic, which he publishes; while another may propose for an entirely different technic the stain Hoffman violet. Then a third laboratory worker may read both articles and wish to try both methods; so he accordingly

orders both dahlia and Hoffman violet. His dealer, who is probably quite unacquainted with dyes, will very likely send him a bottle bearing each name, and the purchaser has no easy way of discovering that the two are identical; so he may continue for years to use the two stains for different purposes, misled by their labels and thinking them distinct. The manufacturers and dealers in stains have sometimes encouraged this confusion by their practice of taking care to have the label on the bottle agree with the name used in the customer's order, regardless as to what the usual name for the dye may be.

An attempt to relieve this confusion has been made by the Commission on Standardization of Biological Stains (1923f) by publishing a list of biological stains with their best known synonyms. In each case one of the names is listed as a preferred designation. Sometimes general usage made it easy to select one name as the preferred one; but in other instances the selection was more or less arbitrary. This same list, with a few revisions in the way of additions and corrections, is given in the appendix of this book (p. 170; see p. 106 of first edition). A few new stains have been added to this list, but essentially it is the same as published in 1923. The chief changes have been in the list of synonyms, which has been revised to omit names that are obsolete and have no present meaning. With but one or two exceptions the preferred designations are still the same as in the first list.

DYE INDEXES

Inasmuch as the dye industry originated in Germany and until the war was almost a monopoly of that country, it is natural that the first serious efforts to index the dyes should have been undertaken in that country. Until recently the only important index of dyes was Schultz's Farbstofftabellen, which is now in its sixth edition (1923). This index lists all of the important textile dyes, giving their synonymy, their chemical composition, methods of preparation, and distinctive characteristics. As these descriptions are concise, it seemed well to refer to the Schultz number of all the stains listed in the article on stain nomenclature above mentioned (Commission, 1923f), wherever such could be given.

More recently another dye index has been published in England by the Society of Dyers and Colourists (1923). This publication, known as the Colour Index, is more complete than even the sixth edition of Schultz, and lists even such dyes as narcein, thionin and iodine green, which are no longer of use in the textile industry and have been omitted from recent editions of Schultz. The synonymy is more complete and up-to-date than that in Schultz, and many more chemical formulae are given. Accordingly in the following pages the stains are denoted by their Colour Index number (abbreviated C. I. No.) instead of by their Schultz number, as in the

list previously published. The Schultz number of each of them is given for reference purposes, however, in the list in the appendix, p. 170.

DYE SOLUBILITIES

Textile dyes are never of a high degree of purity. Some of the impurities are accidental; others are added intentionally so that dyers can obtain the desired shade without having to measure out dyes in very small quantities. Inasmuch as the early biological stains were textile dyes without much, if any, modification, it is natural that some of them should also have been of low dye content, and also that different batches should have been of various degrees of purity. In general the post-war dyes are much more pure than those available before the war. This makes it difficult to prepare stain solutions identical in strength with those prepared before the war.

There are two general types of stain formulae: in one a definite weight of dry dye is specified; in the other a certain volume of a saturated (generally alcoholic) solution of the dye. Each type of formula has its own possibilities of error; and to appreciate the problem it is necessary to understand certain facts in regard to the solubilities of dyes.

The error inherent in the first type of formula is plain at a glance. If two different staining solutions are made up containing 1 g. per 100 cc. of dry methylene blue, and in one case the actual dye content of the dry stain is 90 percent, while in the other only 55 percent (a difference actually observed in samples on the market), it is plain that the two solutions must differ greatly in their strength. For this reason an early recommendation of the Commission (1923b) was that formulae of the second type be preferred, on the assumption that a saturated solution of a dye would be more likely to be of constant dye content than different lots of dry stain bought in the market.

This recommendation, however, was made without complete understanding of the actual facts of the case. The amount of a dye that will go into solution in either water or alcohol depends upon the amount of mineral salts present. If a dye contains a large percentage of sodium chloride, for instance, a saturated solution will be of considerably lower actual dye content than if the dye were free or nearly free from salt; the sodium chloride prevents the solvent from taking up as much of the dye as it would normally. For this reason two staining solutions each containing 10 percent by volume of a saturated solution of the two methylene blues above mentioned would be quite different from each other in actual dye content, altho possibly more nearly alike than if they had been prepared with identical weights of the dry stain.

As soon as these facts were fully understood, the Commission (1923e) modified its recommendation. It is plain that the only way

two staining solutions can be made identical if different batches of stain are used is to make them up on the basis of the weight of *actual dye* present in the stain employed. This can be done only if the manufacturer has coöperated to the extent of printing the actual dye content of each batch of stain on the container in which it is sold. At present the Commission is issuing its certification only if the dye content is printed on the label. In this way it is hoped that eventually all stains on the market will be so marked; and then when staining formulae are readjusted so as to call for definite quantities of actual dye, the preparation of staining solutions will be put on a more scientific basis.

In the present edition of this book there are listed, both under the individual dyes and in a table on pp. 199–200, the solubilities in alcohol and water at 26° of the most commonly used stains. These data have been published recently by Holmes (1927, 1928, 1929) and are based upon determinations made in the Color laboratory of the Department of Agriculture. Obtaining the information was found to be a matter of some difficulty because of the profound effect of impurities upon the amount of a dye capable of going into solution. This same fact must be taken into account in interpreting the figures. To obtain a solution of any dye as strong as the figures here given would indicate to be possible, the dye sample employed must be strictly pure; and as impurities are always present, no user of stains can expect to obtain a saturated solution of the theoretically possible strength. These data, nevertheless, should be of use in indicating roughly how much of any stain to employ to obtain a saturated solution.

INFLUENCE OF IMPURITIES ON INTENSITY OF STAINING

Impurities present in any dye sample or in the solvent not only influence the solubility of the dye but may have a great effect on the intensity of staining. An impurity present may alter the H-ion concentration of the staining fluid; and acid dyes stain better in more acid solutions, basic dyes in more alkaline solutions. If, moreover, the impurity is a mineral salt or the salt of an organic acid, it may have an effect on intensity of staining even if the reaction of the solution is not altered. Theories to explain these facts are discussed in Chapter X.

The practical lesson from this is that poor staining results with any dye sample are as often to be explained by the presence (or absence) of impurities in the dye, or in the water used as a solvent, as by some irregularity in the dye itself. In many instances the impurities normally present in some dye may be necessary to assure its proper behavior as a stain; and too great effort in the way of purification may be detrimental. This seems to have been unquestionably the case in respect to rose bengal. It has been found (Conn and Holmes, 1928) that a sample of the latter dye,

too highly purified for good results if dissolved in distilled water, may stain satisfactorily in tap water containing a certain amount of calcium. It may well happen that the user of some dye which gives too weak staining can increase its intensity of action by adding minute amounts of some mineral salt to the staining solution or by altering the H-ion concentration. In trying this experiment great caution must be employed, for large effects are sometimes produced by minute changes, and it is easy to bring about too weak staining on the one hand, or overstaining on the other if too much of any salt, acid, or base be added.

CHAPTER III

THE SPECTROPHOTOMETRIC ANALYSIS OF DYES

CHEMICAL methods alone are inadequate in the analysis of many dyes. Not only is the detailed chemistry of some dyes obscure, but the reactions are often complicated by adulterating dyestuffs in such a manner as to preclude entire reliance on rigorous chemical methods. Often a slight change in the arrangement of atoms within the molecule will make a marked change in the nature of the dye, while such a minor change in structure is not always readily detected by chemical means alone. Hence the advisability of employing certain physico-chemical characteristics as displayed by the spectrophotometer in the study of dyes. This method also is rapid and convenient.

The absorptive spectra obtained by the spectrophotometer are usually characteristic of any particular dye. It has recently been shown, in fact, that quantitative as well as qualitative data may be obtained by the spectrophotometer; and it is apparent that the combination of qualitative and quantitative results may easily differentiate nearly all dyes, even tho differing from each other only in very minor particulars of chemical structure. So important is this method of study that a discussion of the principles involved is necessary here.

To understand these principles it must be recalled that when a ray of light passes thru a prism it is refracted into many rays differing from each other in wave length and in color. Now the color of any substance arises from the selective absorption or reflection of definite parts of the visible spectrum, as light passes thru or is reflected from the substance. In the visible spectrum is included light of wave lengths intermediate between about 400 and 725 millimicrons. (The millimicron, denoted by the symbol $m\mu$, is 0.001 μ or 0.000,001 m.m in length.) The color of the light in the spectrum varies, with increasing wave length, from violet to red, appearing blue at about 450 $m\mu$, green at about 500 $m\mu$, yellow at about 550$m\mu$, and orange at about 600 $m\mu$, as shown in Fig. 1. The color of light which reaches the eye after transmission thru or reflection from a colored substance is complementary to the color of the light absorbed by that substance. A violet dye, for example, appears violet because of its predominent absorption of yellow light. In Fig. 1, the complementary colors corresponding to the various parts of the spectrum are also shown beneath the colors of the spectrum.

The color of substances is ordinarily of complex origin, depending

upon the absorption of light in varying degrees, over an extensive spectral range. Whereas the unaided eye is able to register only the composite effect, it is possible to resolve this effect into its component factors with the aid of a spectrophotometer. Altho the eye is unable to distinguish between a violet dye and a suitable mixture of a red and a blue dye, the heterogeneous character of the mixture is readily apparent upon spectrophotometric examination. Pure dyes may have simple absorption spectra, in that their light absorption is all at one part of the spectrum, or they may be more complex, showing two or more points on the spectrum at each of which light is absorbed to greater extent than on either side of it. Thus even in the instance of pure products of identical color to the eye, the spectrophotometer frequently reveals decided differences when the character of the light absorption is considered in detail.

The essential principle of spectrophotometric analysis may be understood by reference to Fig. 2, which is a diagram of a spectrophotometer. Two parallel beams of light of equal intensity enter

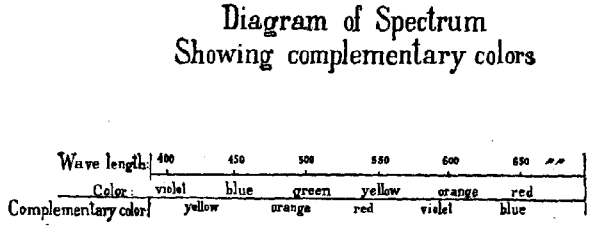


FIG. 1. Diagram of spectrum

the photometer box by separate orifices, pass thru a prism where they are resolved into visible spectra, and then reach the eye in contiguous fields so that very accurate comparison between the two spectra is possible. The arrangement is such that one beam passes directly to the prism whereas the intensity of the second beam may be reduced in any desired proportion by revolving the photometer circle. A glass cell containing a dilute solution of the dye to be examined is interposed in the path of the first beam and a similar cell containing water (or whatever solvent is used in the case of the dye) in the path of the second beam. The spectrum of the beam which has passed thru the dye solution will be found deficient in those portions which have been absorbed by the dye: and the degree of the deficiency at any position in the spectrum may be measured by determining the degree to which the intensity of the light of the second spectrum must be reduced in order to obtain an equal intensity in the two fields observed by the eye.

The shutter of the eyepiece may be partially closed so that only a narrow spectral range is visible; this allows the eye to concentrate on the matching of two small fields, each of which appears uniform in color. The instrument is provided with a screw drum, calibrated in wave lengths, by means of which the prism may be rotated in such a manner as to bring light of any desired wave length into the center of the field of vision.

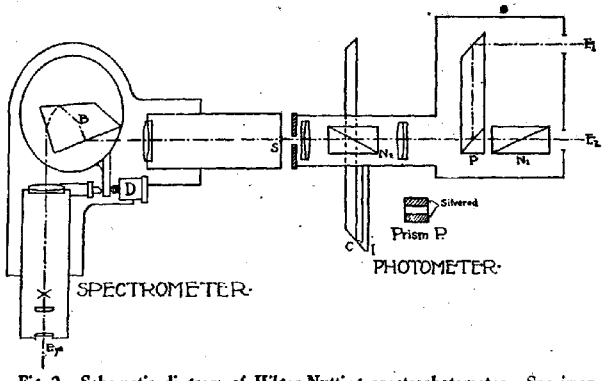


Fig. 2. Schematic diagram of Hilger-Nutting spectrophotometer. Specimen holder with dye dissolved in a suitable solvent is placed at E_1 while a similar cell with solvent alone is placed at E_2. Both are equally illuminated from the same light source. Thru the partially silvered prism, P, the upper and lower portion of the photometer field is illuminated thru the specimen while the central portion is illuminated thru solvent alone. This latter illumination may be varied by the rotation of the analyzer, N_2, which is actuated by a graduated circle C, reading with the index I. By this means the illumination of the several portions of the field is maintained equal and absorption values read off of circle C. The spectrometer of the constant deviation type serves to isolate particular portions of the spectrum by means of a graduated drum D which thru a screw actuates the prism table supporting prism B.

The photometer may be one of several types, the Nutting, Martens, or improved Martens, all of which are optical and depend upon polarization for varying the light, or of the so-called sector or mechanical types. The improved Martens is the most convenient as it is graduated to read direct in per cent transmission, and absorptive indices (Bunsen extinction coefficient, **k**), or indirectly in angular rotation of the analyzing Nicol from which any of the above data may be computed. This type also permits reversal of specimen and solvent, which is necessary to remove possible errors due to polarization within the sample or solvent if accurate results are to be obtained. Convenience and accuracy require an instrument capable of direct reading and reversal, which is practicable only with the improved Martens.

In measuring the complete visible absorption of a dye, a series of measurements is made over the portion of the spectrum in which any appreciable absorption may be noted. This is done by

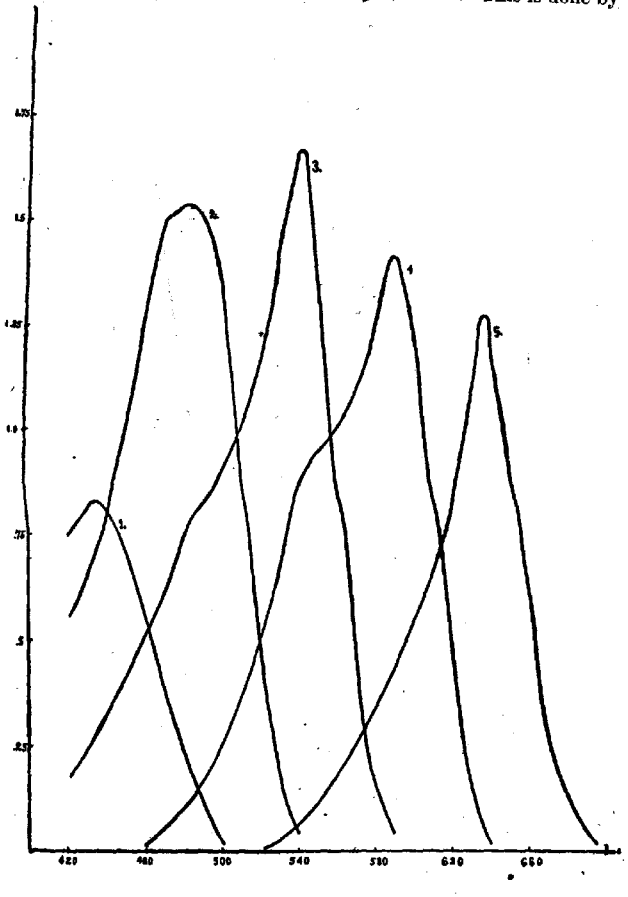


FIG. 3. Absorption curves of five dyes of different colors:

1. Tartrazine (yellow)
2. Orange G.
3. Fuchsin (red)
4. Crystal violet
5. Neptune blue BG.

setting the drum at some definite wave length and observing whether both beams of light reaching the eye are of the same intensity; if not, the photometer circle is turned until the beam which has not passed thru the dye is of the same intensity as that which has passed thru the dye cell. A reading of the absorptive index is then made. Further readings may be made at intervals

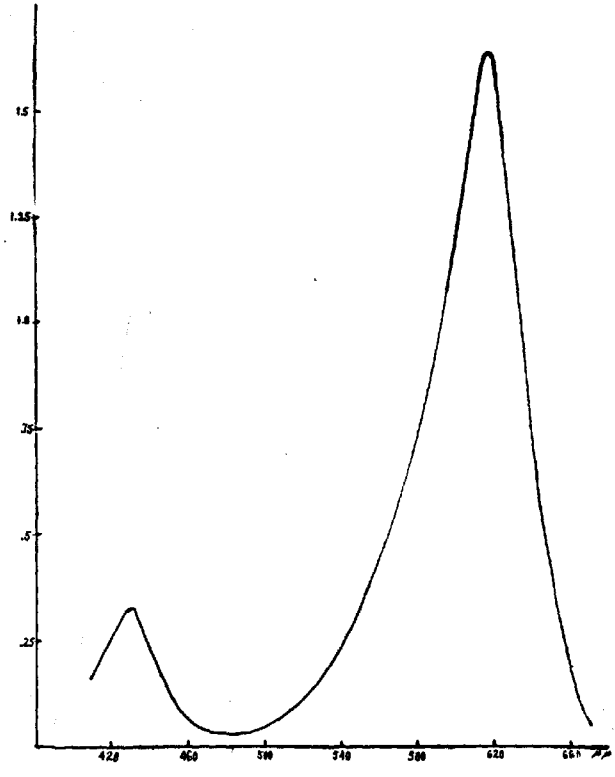


FIG. 4. Absorption curve of malachite green.

of 10mμ, with intermediate determinations in the immediate vicinity of the maximum absorption or at any other point at which it may appear desirable to bring out detail. If absorptive indices are then plotted against wave length a graphic representation of the absorption band of the dye is obtained.

If measurements are carried out under suitable standardized conditions, the spectral position and the general form of the ab-

sorption curve are characteristic of the individual dye, while the magnitude of absorptive indices (the height of the curve) varies directly with the amount of dye present. The absorption curves

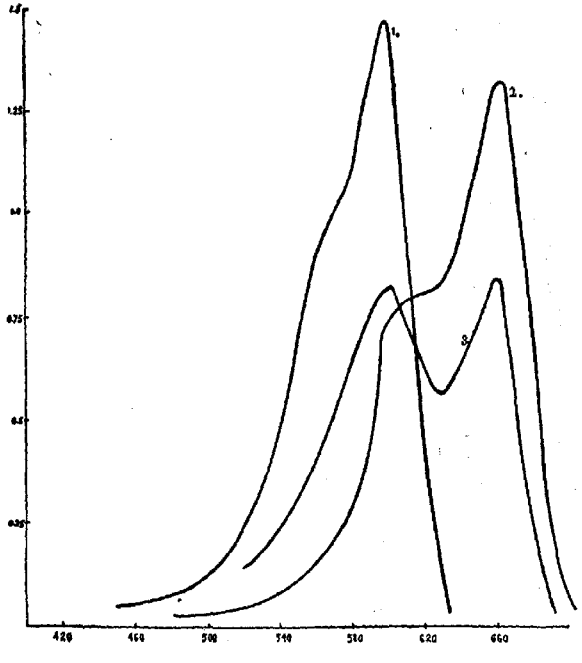


FIG. 5. Absorption curves of:

1. Thionin
2. Methylene blue.
3. Apparent mixture of these two dyes, incorrectly labelled (altho in good faith) a dye intermediate between them in chemical composition.

of dyes which are very closely related in structure are sometimes so similar as to be practically identical. In such instances the individual dyes may be recognized by means of quantitative determinations of the degree in which their absorption is modified under the influence of suitable variations in conditions.

The absorption curves of typical yellow, orange, red, violet, and blue dyes are recorded in Figure 3. It will be noted that their maximum absorption in each case falls within the range of the complementary color (cf. Fig. 1). The great majority of dyes of these

colors, in the usual solvents and under the usual conditions, show but one absorption band in the visible spectrum. The curves are seldom perfectly symmetrical, however, and usually give indications of localized secondary absorption in some portion of the band. It has been shown that this secondary absorption is due, in numerous instances, to a tautomeric form of the dye. It should never be accepted as evidence of the presence of a second dye unless it has been ascertained that it is not found with a pure sample of the dye under conditions of examination.

The absorption curve of a green dye is recorded in Fig. 4. It has a principal band in the red and a secondary band in the violet. Both the absorption curve and the color of the dye could be matched closely by mixing a suitable blue and a yellow dye in the correct proportions. All green dyes absorb appreciable amounts of violet light as well as of red light.

In Fig. 5 is given the absorption curve of a dye mixture, together with the curves of the component dyes. The mixture is reported to have been marketed in good faith as asymmetrical dimethyl thionin, a dye which is intermediate in constitution and in color between thionin and methylene blue (see methylene azure p. 62). The absorption curve plainly indicates the presence of two dyes, and suggests their probable identity. (It would be advisable to effect the separation of small amounts of both dyes, if their positive identification is desired.) The color of the mixture is very similar to that of dimethyl thionin. The absorption curve of that dye, however, is a simple and well defined curve resembling those of thionin and methylene blue, but occupying an intermediate position in the spectrum.

This illustration shows how valuable the spectrophotometric analysis may be in determining whether a given product is a simple dye or a mixture of two or more dyes. This fact, together with its use in determining the exact shade of any dye, makes it the most valuable test to apply to a stain, other than to determine by actual use whether the sample will prove satisfactory to the microscopist or not.

The user of biological stains may well question, however, whether spectrophotometric data have any actual value for him in guiding him in the use of dyes. Such data as are given in this book do in fact have a distinct use, provided he understands the principle underlying them. Let us suppose for example that never having heard of pyronin, he did not know its color; in such a case the absorption maximum (545) as given on p. 118 would furnish him this information. By referring to the diagram of the spectrum on p. 33 he would find that an absorption maximum at 545 means a primary absorption in the yellows; and as the complementary

color is red, he would thus learn that pyronin is a red dye. In a similar way if he were trying to decide between using one or the other of two closely related dyes knowledge of their absorption maxima might help him, provided the decision depended on shade. Thus if he were using azure A and obtained a shade somewhat too red, the spectrophotometric data given on pp. 61–64 would show him whether to substitute azure B or azure C. It will be seen that the absorption maxima of azures A, B, and C are respectively 638, 652, and 611. Reference again to the diagram on p. 33 shows that the complementary color corresponding to wave-length 652 would be on the blue side of 638, while 611 would be on the red side; accordingly azure B should be selected if bluer staining than with azure A were desired.

CHAPTER IV

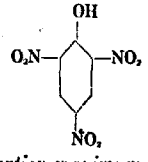
DYES OF THE NITRO, AZO, AND OXYQUINONE GROUPS

1. THE NITRO GROUP

In this group the chromophore is $-NO_2$. The chromophore is of such a strongly acid character that the dyes of this group are all acid dyes. The best known nitro dye is picric acid.

PICRIC ACID C. I. NO. 7*

Picric acid is formed by the action of nitric acid on phenol, thus introducing three nitro groups:



(An acid dye; absorption maximum about 360 in alcohol)

Solubility at 26°C: in water 1.18%; in alcohol 8.96%

This compound forms salts by the dissociation of the –OH group, and the salts have considerable value as stains. Ammonium picrate is the most commonly thus used.

Picric acid (or one of its salts) is quite extensively employed in contrast to acid fuchsin in the Van Gieson connective tissue stain. It is also used as a general cytoplasmic stain in contrast to the basic dyes. It has further application as a fixative for tissues that are to be sectioned and as a slow decalcifying agent.†

Van Gieson's connective tissue stain: The proportions given are those recommended by Mallory and Wright (1924, p. 119). Occasionally it will be found necessary to increase the proportion of the acid fuchsin.

1. Harden in chrome salts or in corrosive sublimate. The results after alcohol are not so good.
2. Stain deeply in alum-hematoxylin, or Weigert's iron-hematoxylin (see pp. 158–9).
3. Wash in water.
4. Stain for three to five minutes in a mixture of:

*This abbreviation stands for the number in the "Colour Index;" see Chapter III, p. 28.

†For bibliographic references concerning the procedures referred to in this chapter see Table 2 in Appendix I, pp. 174–195, and also the bibliography in Appendix III, p. 204.

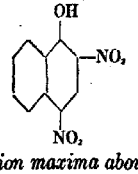
1%. Aqu. Sol. acid fuchsin,* 5 cc.
Sat. Aqu. Sol. picric acid, 100 cc.

5. Dehydrate in 95% alcohol.
6. Oleum origani cretici.
7. Xylol colophonium or balsam.

MARTIUS YELLOW C. I. NO. 9

Synonyms: *Manchester yellow, Naphthol yellow.*

This dye is usually the sodium, or sometimes calcium or ammonium, salt of the following acid:



(An acid dye; absorption maxima about 445, [399, 379])
Solubility of sodium salt at 26°C: in water 4.57%; in alcohol 0.16%. Solubility of calcium salt: in water 0.05%; in alcohol 1.90%

Martius yellow has been used by Pianese in combination with malachite green and acid fuchsin for studying cancer tissue; the same technic was applied to plant tissue by Müller, and is now quite extensively used by plant pathologists in studying sections of tissue infected by fungi. The dye is also used in preparing certain light filters used in photomicrography.

Pianese IIIb stain:

Martius yellow†	0.01	g.
Malachite green	0.50	g.
Acid fuchsin	0.10	g.
Distilled water	150	cc.
Alcohol, ethyl, 95%	50	cc.

For use with plant tissues, wash material in water or alcohol, stain in above staining fluid 15–45 minutes, remove excess stain in water, and decolorize in 95% ethyl alcohol, acidified with a few drops of hydrochloric acid. Host tissue stains green and mycelium a deep pink.

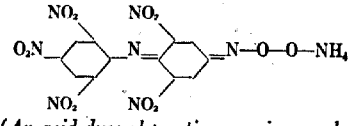
*This should preferably be of the type discussed by Scanlan, Holmes and French (1927). See under this dye, p. 99.

†Little information is available as to the exact nature and dye contents of the three dyes required in this technic. Good results have been obtained with Commission certified martius yellow and acid fuchsin.

AURANTIA C. I. NO. 12

Synonym: *Imperial yellow.*

This dye is the ammonium salt of hexanitro-diphenylamine.



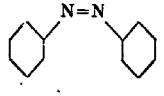
(An acid dye; absorption maximum about 425)

Solubility at 26°C: in water nil; in alcohol 0.33%

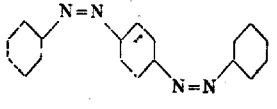
It is obsolete as a textile dye and is almost unknown as a biological stain. Possibly the dye is not at present manufactured. It is called for, however, in combination with toluidine blue and acid fuchsin in the Champy-Kull technic for demonstrating certain cell constituents (mitochondria, etc.)

2. THE AZO GROUP

The azo dyes are characterized by the chromophore —N=N— joining benzene or naphthalene rings, thus:

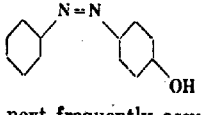


It is possible for the azo group to occur more than once in a molecule, forming the disazo dyes, thus:



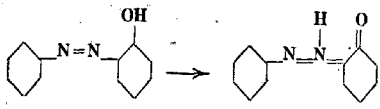
The azo chromophore is distinctly basic; but not sufficiently so to make the dyes basic when they contain hydroxyl radicals. Those containing amidogen radicals are, of course, pronouncedly basic.

The position of the hydroxyl or amidogen group on a benzene ring in relation to the azo group is important. Ordinarily they are in the para position to each other, thus:

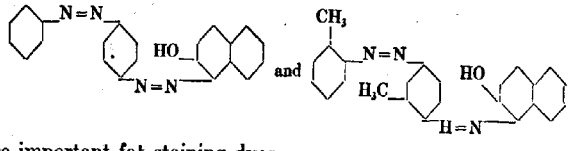


The ortho position is next frequently assumed; rarely the meta position. When the hydroxyl group assumes the ortho position the

character of the compound is quite distinct from that of the para compounds. By a rearrangement of the atoms such a compound is sure to change to a quinoid form, thus:



A compound of this latter structure cannot form salts and does not act as an ordinary dye. It does, however, prove to be soluble in oil and is able to color it by an apparently physical process. Hence the azo-ortho-phenols, or azo-beta-naphthols, like Sudan III and Sudan IV,

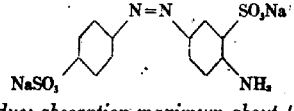


are important fat staining dyes.

FAST YELLOW C. I. NO. 16

(*Echt Gelb*)

Synonym: *Acid yellow.*



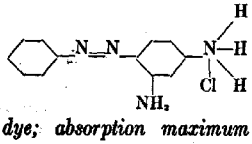
(*An acid dye; absorption maximum about 490 in acid solution*)

Solubility at 26°C: in water 18.40%; in alcohol 0.24%.

This dye is rarely used as a biological stain, but is called for by Schaffer for staining sections of bone, and by Unna in certain stain mixtures used in studying the phenomenon called by him chromolysis.

CHRYSOIDIN Y. C. I. NO. 20

Synonyms: *Brown salt R.* *Dark brown salt R.*



(*A basic dye; absorption maximum about 461*)

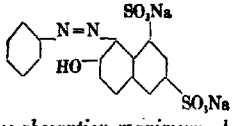
Solubility at 26°C: in water 0.86%; in alcohol 2.21%.

This dye is a good substitute, in some procedures, for Bismarck brown. Like the latter, it is easily injured by heat, and boiling should be avoided in preparing solutions.

ORANGE G. C. I. NO. 27

Synonym: *Wool orange 2G.*

Slightly different grade: *Orange GG, GMP.*



(An acid dye; absorption maximum about 485)

Solubility at 26°C: in water 10.86%; in alcohol 0.22%

This dye is strongly acid because of the two sulfonic groups. It is one of the most valuable plasma stains in histological work. It has great use as a background stain for hematoxylin and other nuclear dyes in cytology. It is frequently employed, both by botanists and zoologists, as a cytoplasmic stain, together with the two nuclear dyes safranin and gentian violet in the Flemming triple stain. It is of importance to the pathologist for its use with anilin blue and acid fuchsin in the Mallory connective tissue stain; and is used in various other double and triple staining methods, such as that of Ehrlich-Biondi-Heidenhain, in which it is mixed with methyl green and acid fuchsin. The Ehrlich "triacid mixture," also a combination of these same three dyes, is used in staining blood. A further use is Bensley's "neutral gentian," a combination of orange G and gentian violet for staining the A and B cells in the islands of Langerhans.

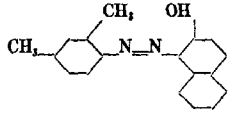
Formula for use as general counterstain:

Orange G (dye content 80–85%.)	1 g.
Distilled water	100 cc.

For the technic by which it is employed in the Flemming triple stain and the Mallory connective tissue stain, see under safranin, p. 81, and anilin blue (water soluble), p. 114, respectively.

OIL RED O C. I. NO. 73

Synonyms: *Sudan II. Oil scarlet. Fast oil orange II. Red B. Fat ponceau. Orange RR.*



(An acid dye; absorption maxima in alcohol: 531.5, 494.5)
Solubility at 26°C: in water nil; in alcohol 0.39%)

A fat-stain recently recommended by French as a substitute for Sudan III, as it has a greater depth of color. It may be employed in the Herxheimer formula (see p. 50), or in the pyridin formula lately proposed by Proescher. The Proescher procedure follows:

Proescher's oil-red-pyridin stain for fat:

Oil red O	about 3–5 g.
70% pyridin (i. e. 70 parts to 30 parts distilled water)	100 cc.

Allow to stand for an hour at room temperature with occasional stirring; it is then saturated and is ready for use. The solution should be kept in a glass stoppered bottle, protected from light and should be filtered before use.

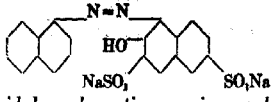
The staining dishes should be well covered to prevent undue evaporation of the pyridin. The stain is to be applied to frozen sections of tissue, fixed in formalin, Mueller-formalin, or formalin-picric-acid, according o the following schedule:

1. Immerse sections in 50% pyridin for about 3–5 minutes.
2. Transfer to oil-red-pyridin for 3–5 minutes. In the case of sections of central nervous system stain 20–30 minutes.
3. Differentiate in 50% pyridin several minutes.
4. Transfer to water.
5. Counterstain with Delafield's hematoxylin (see p. 158) 2–3 minutes. In the case of sections of central nervous system, an acidified solution is recommended, to prevent overstaining of the cytoplasm; this is prepared by adding 2 cc. of glacial acetic acid to 16 cc. of Delafield's hematoxylin.
6. Mount in gum acacia or levulose syrup.

BORDEAUX RED C. I. NO. 88

Synonyms: *Fast red B* or *P. Cerasin. Archelline 2B. Azo-bordeaux. Acid bordeaux.*

Various grades denoted: *Bordeaux B, BL, G, R extra.*



(An acid dye; absorption maximum about 520)

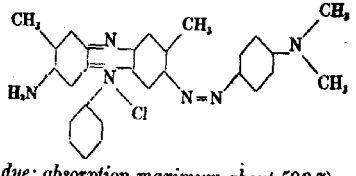
Solubility at 26°C: in water 3.83%; in alcohol 0.19%

Bordeaux red is used as a cytoplasmic stain, as, for example, in aqueous solution when Heidenhain's hematoxylin is to be used immediately afterward as a nuclear stain. It has also been used by Gråberg with thionin and methyl green for staining sections, particularly of spleen, testis, and liver.

JANUS GREEN B. C. I. NO. 133

Synonym: *Diazin green.*

This is an azo dye having an azin as well as an azo chromophore group, and is thus related to the safranins. It is a compound of dimethyl safranin with dimethyl anilin thru an azo group.



(A basic dye; absorption maximum about 592.7)

Solubility at 26°C: in water 5.18%; in alcohol 1.12%

Janus green is best known for its use in demonstrating chondriosomes, stained *intra vitam*, according to the technic of Michaelis, and as more recently developed by Cowdry and Bensley. It is also used by Faris with neutral red for sections of embryos.

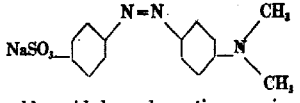
Soep 1927 proposes it in place of methylene blue for studying reductase production in milk.

As vital stain for mitochondria:

Janus green B (55–65% dye content).....0.001 to 0.01 g.
Physiological saline solution.....100 cc.

METHYL ORANGE C. I. NO. 142

Synomyms: *Orange III. Helianthin. Gold orange. Trapaeolin D.*



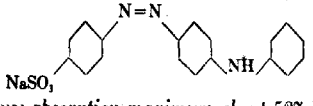
(A weakly acid dye; absorption maximum about 506 in acid solution)

Solubility at 26°C: in water 0.52%; in alcohol 0.08%

This dye has little use as a stain, but is widely employed as an indicator, as it is red in acid, and orange in alkaline solutions. Its chief value as an indicator is that it is sensitive to mineral acids without being affected by carbonates or most organic acids. It has been used by Bergonzini in the place of orange G in the Ehrlich-Biondi stain; and by Ebbinghaus for staining keratin in sections of skin. It is employed in determining the reaction of cell sap in plants.

ORANGE IV. C. I. NO. 143

Synonyms: *Orange N. Acid yellow D. Tropaeolin OO.*

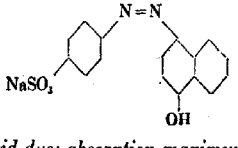


(An acid dye; absorption maximum about 527 in acid solution)

The only biological use of this dye seems to be occasionally as an indicator.

ORANGE I. C. I. NO. 150

Synonyms: *Naphthol orange. Tropaeolin G.* or *000 No. 1.*



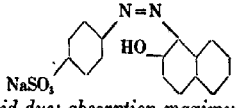
(An acid dye; absorption maximum about 476)

Solubility at 26°C: in water 5.17%; in alcohol 0.64%.

This is another dye which is turned red by excess of alkali and has therefore some use as an indicator.

ORANGE II. C. I. NO. 151

Synonyms: *Gold orange. Orange A, P,* or *R. Acid orange. Orange extra. Mandarin G. Tropaeolin 000 No. 2.*

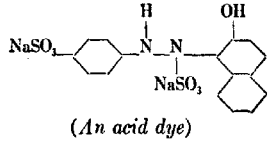


(An acid dye; absorption maximum about 490)

Solubility at 26°C: in water 11.37%; in alcohol 0.15%

This dye, which differs from orange I only in the position of the hydroxyl group on the naphthalene radical, is similar to it in color and properties, but does not change color with changing reaction of its solution. This dye is rarely employed in microscopic work, and yet may be recommended as a valuable substitute for orange G when a stronger yellow is desired for contrast purposes. Thus French uses it in combination with eosin and azure C in a general tissue stain; the technic of this procedure is given under azure C, p. 62.

NARCEIN C. I. NO. 152



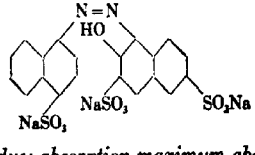
(An acid dye)

Solubility at 26°C: in water 10.02%; in alcohol 0.06%

This dye is a derivative of orange II, prepared from the latter by treatment with sodium bisulfite. It is rarely used either as a textile dye or in microscopic technic. It has been called for by Ehrlich, however, in combination with pyronin and methyl green or methylene blue to form a neutral dye.

AMARANTH C. I. NO. 184

Synonyms: *Naphthol red. Fast red. Bordeaux. Bordeaux SF. Victoria rubin. Azo rubin. Wool red.*

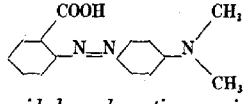


(An acid dye; absorption maximum about 525)

Solubility at 26°C: in water 7.20%; in alcohol 0.01%

Amaranth is not a commonly used stain, but is of considerable importance as a food color. It has been used by Griesbach for staining axis cylinders.

METHYL RED C. I. NO. 211

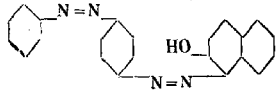


(A weakly acid dye; absorption maximum 530)

This dye does not seem to have been used for staining, but has long been employed as an indicator. Its useful range is from pH 4.4 to pH 6.0 in which it changes from red in acid solutions to colorless in basic. Altho still of value for this purpose it is coming to be replaced by certain of the sulfonphthalein indicators such as brom cresol green and chlor cresol green (see p.138), which are more stable chemically and permit greater accuracy in reading. The chief drawback to methyl red as an indicator is that it is easily reduced with loss of color, and readings must be made very promptly after adding it to the solution to avoid error due to this cause.

SUDAN III. C. I. NO. 248

Synonyms: *Sudan G. Tony red. Scarlet G or B. Fettponceau G. Oil red. Cerasin red.*



(A weakly acid dye; absorption maximum about 641, [590])

Solubility at 26°C: in water nil; in alcohol 0.15%

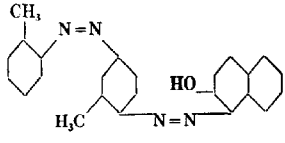
In this dye the hydroxyl group is in the ortho position with respect to the azo group. As explained above (p. 42), such compounds show a tendency toward intramolecular rearrangement so that the hydrogen atom detaches itself from the hydroxyl group and becomes fixed to the neighboring nitrogen. Such a compound is neither acid nor basic, and not being able to form salts is not an ordinary dye, but is fat soluble and has the power of coloring fat. This fact gives Sudan III its chief value to the histologist. It was introduced as a fat stain by Daddi in 1896. It is also employed by botanists together with light green in the technic of Bugnon, for differentiating suberized and cutinized tissue in plants.

Technic for staining fat. This dye when employed today as a fat stain is ordinarily used in the technic given under Sudan IV below.

For some time Sudan III was the only important fat stain known. More is now known in regard to fat soluble stains, thanks to the research of Michaelis (1901). It was he who showed the relation of this property of certain dyes to their lack of basic or acid character. He showed that new dyes with this property and of greater staining power might be built up synthetically by taking advantage of the fact that the azo group will attach itself in the ortho position if the para position is already occupied. In this way azo-ortho-phenols and beta-naphthols can be prepared, and they prove to be fat soluble. Michaelis suggested the following dye, which has now to a considerable extent replaced Sudan III.

SUDAN IV C. I. NO. 258

Synonyms: *Scarlet red. Scharlach R. Oil red IV. Fettponceau. Ponceau 3B.*



(A weakly acid dye; absorption maximum about 657.4, [605.5] in H_2SO_4)

Solubility at 26°C: in water nil; in alcohol 0.09%

This di-azo naphthalene compound is similar to Sudan III except that it is a dimethyl derivative. This fact makes it a deeper, more intense stain; but having the hydroxyl group in the ortho position, it has similar physical properties and is fat soluble. It is, therefore, one of the best fat stains known.

Herxheimer's stain for fat: Use a saturated solution of Sudan IV in:

70% alcohol,.....50 cc.
Pure acetone,.....50 cc.

Staining takes place very quickly and is intense and sharp.

Cover-slip Preparations.—1. Fix in the vapor of formaldehyde for five to ten minutes.

2. Stain in the alcohol-acetone solution of the dye for 2–5 minutes.
3. Dip for an instant in 70% alcohol.
4. Wash in water.
5. Counterstain with alum hematoxylin or methylene-blue.
6. Wash in water.
7. Mount in glycerin or glycerin jelly.

Sections.—1. Make frozen sections of formaldehyde-fixed tissue.

2. Dip for an instant in 70% alcohol.

3. Stain in the alcohol-acetone solution of Sudan IV red for two to five minutes.

4. Wash quickly in 70% alcohol and transfer to water.

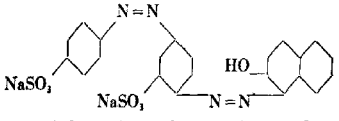
5. Counterstain in alum hematoxylin.

6. Wash thoroly in water.

7. Mount in glycerin or glycerin jelly.

BIEBRICH SCARLET, WATER SOLUBLE C. I. NO. 280

Synonyms: *Croceine scarlet. Scarlet B, or EC. Ponceau B. Double scarlet.*



(*An acid dye; absorption maximum about 503.5*)

The chief biological application of this dye is for medicinal purposes, but it is occasionally used as a plasma stain, notably for tissues after staining with polychrome methylene blue or Unna's hematein. It has also been made use of by Paladino mixed with alum hematoxylin for double staining effect on histological material. In a neutral stain combination with ethyl violet, it has been employed by Bowie for staining the islets of Langerhans.

BISMARCK BROWN Y C. I. NO. 331

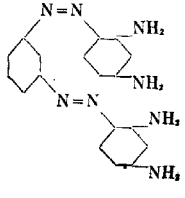
Synonyms: *Vesuvin. Phenylene brown. Manchester brown. Excelsior brown. Leather brown.*

Slightly different shade: *Bismarck brown G.*

To be distinguished from: Bismark brown R or GOOO (C. I. No. 332.)

(*A basic dye. Solubility at 26°C: in water 1.36%; in alcohol 1.08 %*)

The various shades of Bismarck brown are mixtures of different compounds, the most important of which are salts of the following:

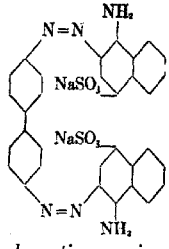


This dye was formerly employed quite extensively as a contrast stain, but has now been replaced to some extent by others. It is still used, however, as a mucin stain, and is good for vital staining and for staining in bulk. It is employed in staining cellulose walls of plants in contrast to hematoxylin; and occasionally for staining bacteria in contrast to gentian violet in the Gram technic.

A caution to observe in connection with Bismarck brown is that solutions should not be boiled before using, because the composition of the dye is changed by heat.

CONGO RED C. I. NO. 370

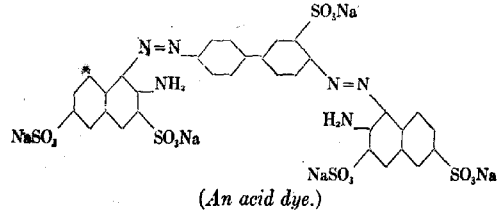
Synonyms: *Congo. Cotton red. Direct red.*



(*An acid dye; absorption maximum about 485.*)

This dye is best known to the biologist as an indicator. The dye acid is blue, but its sodium salt is red. The red color of the salt is readily changed by weak acids into blue. Besides serving as an indicator, congo red has certain histological uses, as for axis cylinders (Griesbach) for embryo sections (Shaffer), for staining elastic tissue (Matsuura) plant mucin, as a stain for Uredineae (Blackman) and as a general background stain in contrast to hematoxylin and other nuclear dyes. It has been used by Klebs as a reagent for cellulose.

TRYPAN RED C. I. NO. 438

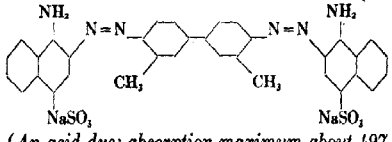


(*An acid dye.*)

The chief use of this dye is as a vital stain.

BENZOPURBIN 4B C. I. NO. 448

Synonyms: *Cotton red 4B. Dianil red 4C. Diamin red 4B. Sultan 4B. Direct red 4B.*

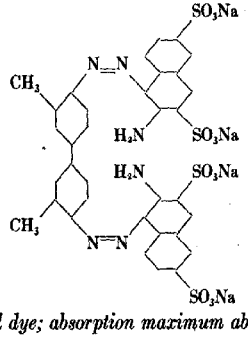


(*An acid dye; absorption maximum about 497.*)

This dye has also been used for vital staining; and has been employed by Zschokke as a plasma stain especially in contrast to hematoxylin.

VITAL RED C. I. NO. 456

Synonyms: *Brilliant Congo R. Brilliant Congo red R. Acid Congo R. Azidine scarlet R. Brilliant Dianil red R*

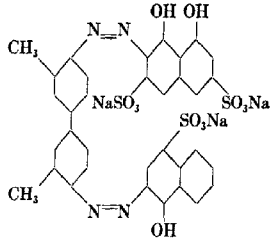


(*An acid dye; absorption maximum about 498*)

This dye is a very important vital stain, much used by Evans. (See Dawson, Evans and Whipple, 1920). Commercially it is better known under the name of brilliant congo R. Difficulty has been found in securing a satisfactory product; but it has been overcome due to the coöperation of the Color Laboratory of the Dept. of Agriculture, and a good vital red is now obtainable from stain dealers.

DIANIL BLUE 2R C. I. NO. 465

Synonyms: *Direct steel blue BB. Benzo new blue 2B, Naphthamine brilliant blue 2R.*

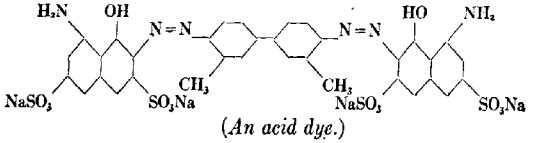


(*An acid dye; absorption maximum about 468*)

This has been used as a vital stain by Corner and Hurni (1918); also by Sutter.

TRYPAN BLUE C. I. NO. 477

Synonyms: *Chlorazol blue 3B. Benzo blue 3B. Dianil blue H3G. Congo blue 3B. Naphthamine blue 3BX. Benzamine blue 3B. Azidine blue 3B. Niagara blue 3B.*



(*An acid dye.*)

This is a valuable vital stain. By injection into the circulatory system, it is employed for staining the uriniferous tubules; also for the study of histiocytes and Kuppfer cells.

Other azo dyes sometimes mentioned in connection with histology are:

Janus red; C. I. No. 266.

Tropaeolin O; C. I. No. 148. Syn: *Chrysoin. Gold yellow. Acid yellow.*

Tropaeolin Y; C. I. No. 148 (see note).

Roccellin; C. I. No. 176. Syn: *Fast red A, AV,* or *O. Cerasin, Rubidin. Cardinal red.*

Crystal ponceau 6R; C. I. No. 89; Syn: *Ponceau 6R.*

Carmin naphtha; C. I. No. 24. Syn: *Sudan 8. Scharlach B. Oil yellow.*

Alizarin yellow GG; C. I. No. 36. Syn: *Anthracene yellow. Benzene yellow.*

Chrysoidin R; C. I. No. 21. Syn: *Cotton orange. Cerotin orange.*

Alizarin yellow R; C. I. No. 40. Syn: *Alizarin orange. Benzene yellow PN. Orange R. Anthracene yellow RN.*

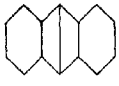
Diamond flavine; C. I. No. 110.

Diamond black F; C. I. No. 299. Syn.: *Salicin black. Chrome black.*

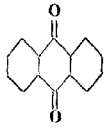
Niagara blue 4B; C. I. No. 520. Syn.: *Niagara sky blue. Benzoin sky blue. Dianil blue H6 G. Congo sky blue. Naphthamine blue.*

3. THE OXYQUINONE GROUP

The oxyquinone dyes include derivatives of anthracene,

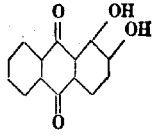


thru its oxidation product anthraquinone:



These dyes are the first to be considered here in which the quinoid structure occurs. The quinoid ring, which is the most important chromophore in nearly all the dyes to be discussed in the three following chapters, forms very strong chromogens, which require only the addition of auxochrome groups to be converted into strong dyes, either basic or acid. The chromogen anthraquinone is converted into a dye by the addition of hydroxyl groups, its best known derivatives among the dyes being: 1:2 dihydroxy-anthraquinone (alizarin) and 1:2:4 trihydroxy-anthraquinone (purpurin). Both of these compounds occur in nature in the root of madder, being the colored principles of madder extract. They have the property of combining with metallic oxides to form so-called "lakes," insoluble compounds of different color from the dye entering into them. This makes them valuable ones to use after mordanting with aluminium, iron or chromium compounds.

ALIZARIN C. I. NO. 1027



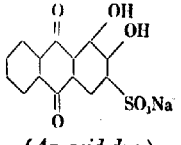
(*An acid dye; absorption maxima about* [*610.8*], *566.5*, [*527.6*] *in alkaline solution.*)

Solubility at 26°C: in water nil; in alcohol 0.125%

Alizarin stains tissues a feeble yellowish red if used on them directly. In the presence of aluminium compounds intense red colors are formed; bluish violet in the presence of iron; and brownish violet in the presence of chromium. It has been used as a stain for nervous tissue. The chief present use of alizarin, however, is as an indicator.

ALIZARIN RED S C. I. NO. 1034

Synonyms: *Alizarin red, water soluble. Alizarin carmin. Alizarin sulfate.*



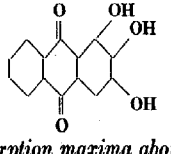
(*An acid dye.*)

Solubility at 26°C: in water 7.69%; in alcohol 0.15%

This dye, sodium alizarin sulfonate, is used by Benda for staining chromatin in combination with crystal violet, the chromatin staining brown, while the mitochondria stain violet. It is also used as a vital stain for nervous tissue in small invertebrates, and by Schrötter for sections of nervous tissue.

PURPURIN C. I. NO. 1037

Synonyms: *Alizarin No. 6. Alizarin purpurin.*



(*An acid dye; absorption maxima about* [*521.1*], *485.5*, [*455.5*] *in alcohol*)

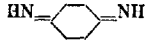
Purpurin is very similar to alizarin, but forms scarlet red lakes with alumina. It has been used as a nuclear stain for histological material, and for determining the presence of insoluble calcium salts in the cell contents.

CHAPTER V

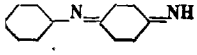
THE QUINONE-IMIDE DYES

THE dyes of the quinone-imide group contain two chromophore groups, the indamin group —N=, and the quinoid benzene ring

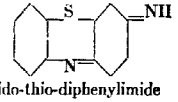
They are derivatives of the theoretical compound paraquinone imide, which, if it existed in its free state, would have the formula



In the typical indamin formula one of the imide hydrogen atoms is replaced by a phenyl group, thus:

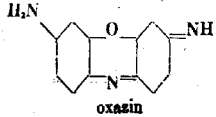


In the thiazins the introduction of a sulfur atom, attached to both the phenyl and the quinone groups, forms a third closed ring, as:



imido-thio-diphenylimide

In the oxazins, an oxygen atom takes the place of the sulfur of the thiazins, thus:



oxazin

1. THE INDAMINS

The indamin dyes are methylated amino derivatives of indamin. No dye in this group is a common biological stain. The following are occasionally mentioned, however, in connection with histology:

Bindschedler's green. A tetramethyl indamin. C. I. No. 819.
Toluylene blue. A diamido, dimethyl indamin. C. I. No. 820.

2. THE THIAZINS

The thiazins constitute one of the most important groups of dyes from the standpoint of the biologist; while for textile dyeing the group contains but a small number of dyes of any importance. In these compounds, as mentioned above, the two benzene rings are further joined by a sulfur atom.

THIONIN C. I. NO. 920

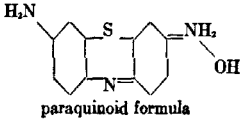
Synonym: *Lauth's violet.*

*(A basic dye; absorption maximum about 602.)**

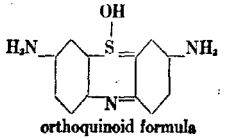
Solubility at 26°C: in water 0.25%; in alcohol 0.25%

Thionin, having two amino groups, is a strongly basic dye. The exact structural formulae of this dye and its derivatives, as well as many others in which two benzene rings are similarly joined, are in some dispute. At least two types of formulae are possible for the thiazins and oxazins, as well as for the xanthene dyes (Chapter VII). One type is known as the orthoquinoid, the other as the paraquinoid.

It is supposed (see p. 18) that when the quinoid ring is formed the two hydrogen atoms replaced by atoms or groups with double valency bonds may be either in the para or in the ortho position to each other. It will also be recalled from elementary chemistry that sulfur and oxygen may be either bivalent or tetravalent. These facts make it possible for a thiazin or an oxazin to have either one or the other of the different structures represented by the following two formulae for the theoretical thionin base:



paraquinoid formula

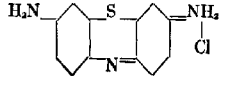


orthoquinoid formula

*See Fig. 5, p. 37.

In the case of the paraquinoid formula the compound is an ammonium base of the type discussed on p. 20, which is capable of salt formation thru its pentavalent nitrogen. In the case of the orthoquinoid formula the salt formation takes place thru the tetravalent sulfur, the base being of the type known as a sulfonium base. There are arguments in favor of either formula, and from the standpoint of the biologist it does not matter which is preferred. Possibly both forms actually exist simultaneously. For the sake of uniformity the paraquinoid form will be shown in the following pages wherever possible; but with the understanding that the orthoquinoid form is equally permissible.

The dye, thionin, is a salt, generally a chloride, of the above mentioned base; and on the assumption of paraquinoid structure, it has the following formula:



Thionin is violet in dilute solution, and has a very pronounced metachromatic effect, the colors in sections stained with it ranging from blue to reddish violet.

It is no longer used as a textile dye, and is very carefully to be distinguished from thionin blue (C. I. No. 926) which is known to the trade and is sometimes furnished in place of the desired dye when thionin is ordered. Thionin is an especially valuable dye for histological work on account of its metachromatic properties, that is its ability to impart different colors to different histological or cytological structures. It is a very valuable chromatin and mucin stain, proving especially useful in staining the tissue of insects; and is recommended by Ehrlich because it stains amyloid blue but mast cells and mucin red. It is a useful vital stain. Perhaps its greatest value at the present time is in the staining of frozen sections of fresh animal or human tissue, particularly in the study of tumors. It is also used by Frost for staining very young bacterial colonies in his "little plate" technic for counting bacteria. (Unfortunately Frost specifies thionin blue in one of his papers, altho the latter proves entirely unsatisfactory for the purpose.)*

Thionin for general nuclear staining:

Thionin (85–90% dye content) ..... 0.5 g.
Ethyl alcohol (20%) ..... 100 cc.

Besides serving as a good nuclear stain for sections of fixed tissue, this solution is satisfactory, as employed by Mallory, in staining frozen sections of

*For bibliographic references concerning the procedures referred to in this chapter, see Table 2 in Appendix I, p. 174, and also the bibliography in Appendix III, p. 204.

fresh tissue, as in the case of biopsies. In the latter technic the sections are immersed in water, placed briefly (1 to 2 minutes) in the stain, washed and mounted in water for examination

Acetic Thionin (Frost):

Thionin*	1 g.
Distilled water (hot)	1200 cc.

Dissolve and filter; then add:

Glacial acetic acid	60 cc.

This staining solution is recommended by its author for staining bacteria in agar films inoculated with milk.

Carbol Thionin (Nicollé):

(Formula quoted from Eyre, 2nd Ed., 1915, p. 92)

Thionin*	1.0 g.
Phenol	2.5 g.
Distilled water	100 cc.

Filter; and dilute before use with an equal quantity of distilled water.

In the source cited above the dye is named thionin blue; but as Lauth's violet is given as a synonym, it is apparent that thionin, not thionin blue, is intended.

This is given by Krause as a useful formula, but has not been tried out in the Commission laboratory.

Frost's "little plate" technic for counting bacteria in milk:

1. Mark off an area of 4 sq. cm. on a microscopic slide with a wax pencil. Sterilize in a flame and place on a level surface maintained at a temperature of 45°C.
2. Place 0.05 cc. of the milk to be examined on the slide, by means of a sterile, accurately calibrated pipette.
3. Add to the milk an equal amount of sterile bacteriological nutrient agar, warmed to 42–45°C. Mix with a sterile platinum needle or loop, and carefully spread over the marked area.
4. Remove to a cool, level surface, and allow the mixture to harden.
5. Incubate in a moist sterile chamber for a few hours, long enough to allow the bacteria to grow into distinct colonies, altho they may not be visible to the naked eye; 8 hours is most satisfactory, altho 4 hours incubation frequently gives good sized colonies, while incubation over night is not too long if more convenient.
6. Dry the preparations quickly and thoroly at a temperature a few degrees under the boiling point of water.
7. Stain with methylene blue, or preferably with acetic thionin (see above), by putting the dried, hot films directly into the staining fluid and keeping them there about 10 minutes.

---

*No definite information is at hand, but presumably thionin of 85–90 % dye content is best in these formulae.

8. Wash in water; and dry again as above.
9. Count the colonies under the microscope.

By this procedure the colonies should be deeply stained while the background is colorless, especially by artificial light. Should the stain be too strong or if made in greater concentration than indicated in the formula, it may be diluted to the proper strength with a 5% solution of glacial acetic acid.

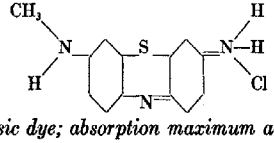
METHYLENE AZURE

This was first recognized as one of the components of polychrome methylene blue by Bernthsen (1885). It is now known to be a mixture, not a simple dye. Definite knowledge of its chemistry has been obtained by Kehrmann (1906), Bernthsen (1906) and MacNeal (1906, 1925); It is regarded as primarily a mixture of azure A and azure B, described below, compounds which Bernthsen and MacNeal have shown simple methods for preparing in relatively pure condition by the oxidation of methylene blue. Another oxidation product of methylene blue, recently described by Holmes and French (1926), has been named azure C. Now that these three dyes, azures A, B and C are recognized, the name methylene azure should be dropped, and users should specify the actual dye employed.

Azure I (Giemsa) is a trade name applied to a secret preparation which appears to be a somewhat variable mixture of azure A and azure B. Azure II (Giemsa) is an intentional mixture of azure I with an equal quantity of methylene blue. Products of these same names are now sold by various companies and they are certainly not all identical with the original Giemsa preparations. They all probably agree, however, in containing azure A as a main constituent; and as this seems to be the most important dye of the group the wisest plan seems to be to order azure A rather than azure I or methylene azure.

AZURE C.

This dye is mono-methyl thionin:



(A basic dye; absorption maximum about 611)

It is not a textile dye; and up to the present time has been manufactured only on a laboratory scale. A small lot, thus prepared, is now on sale by the National Aniline and Chemical Co. This dye, like the other azures described below, has up to the present been

prepared only by the oxidation of methylene blue. If they prove sufficiently valuable, a process of manufacture by direct synthesis will undoubtedly be developed. A product thus manufactured would probably be cheaper as well as purer than the present supply.

Azure C is recommended by French (1926 b) with orange II and eosin as a tissue stain. Haynes (1926 a) finds it possible to obtain equally good results, however, with azure I (i. e. azure A) if the technic is slightly modified. Later (1927) she reports excellent staining with it; but it is still uncertain whether it gives results sufficiently different from thionin on the one hand and azure A on the other to be of decided histological value.

French's tissue stain: To be used only for formalin-fixed material. The following schedule is to be followed:

1. Xylol, 3 minutes
2. Absolute ethyl alcohol, 3 minutes
3. 95% ethyl alcohol, 3 minutes
4. Water, 3 minutes.
5. Azure C (1.5% aqu. sol.), 5 minutes
6. Absolute methyl alcohol, 5–10 seconds.
7. 30–40 seconds in the following solution:

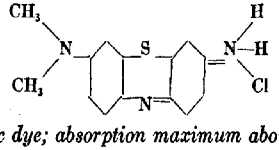
Eosin Y	0.025	g.
Orange II	0.025	g.
Glacial acetic acid	1.0	cc.
Absolute ethyl alcohol	99.	cc.

8. Two changes of absolute alcohol, 1–2 minutes each.
9. Two changes of xylol, 1–2 minutes each.
10. Xylol balsam.

AZURE A.

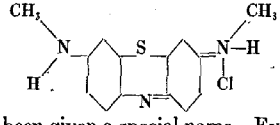
Synonym: *Methylene azure, azure I.*

This is now recognized as asymmetrical dimethyl thionin:



(*A basic dye; absorption maximum about 638*)

It is not a textile dye; but is probably present in much of the methylene blue on the market. It must be distinguished from the isomeric symmetrical compound:



which has never been given a special name. Ever since first recognized by Kehrmann (1906) the impression has been current that this latter dye has no staining value and is of a distinctly different category from the azures. It is difficult to tell how this impression has arisen, but possibly it may have been due to work with an impure dye. Haynes (1927) finds both isomers to have almost identical staining properties.

There is no mistaking the value of azure A; it is in fact regarded by MacNeal (1925) as the most important nuclear staining constituent of polychrome methylene blue. It is called for in all recently proposed formulae for the tetrachrome blood stain (see p. 147). It has been certified by the Commission for some time under the name of methylene azure; and it is proposed in the future to certify it under the more definite name, azure A.

Haynes (1926a) has employed this dye as a nuclear stain preceding eosin; and (1926b) following phloxine in a procedure similar to the Mallory phloxine-methylene-blue technic.

Haynes' modification of French's stain: This may be applied to material fixed in picro-sulfuric, Bouin, or Zenker fluids, or in 95% alcohol with 3% bichromate. Employ the following schedule:

1. Xylol, 3 minutes.
2. Absolute ethyl alcohol, 3 minutes.
3. 95% ethyl alcohol, 3 minutes.
4. Water, 3 minutes.
5. 1.5% aqu. sol. azure A (85% dye content), 5 minutes.
6. Absolute ethyl alcohol, 5–10 seconds.
7. Sat. sol. of ethyl eosin (C. I. No 770) in clove oil, 30 seconds.
8. Xylol, 10–30 seconds.
9. Xylol, two changes of 1–2 minutes each.
10. Mount in xylol balsam.

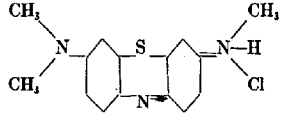
Haynes' phloxine-azure tissue stain: A modification of Mallory's phloxine-methylene-blue stain. May be used following Bouin or Zenker fixation.

1. 2.5% aqu. sol. phloxine (dye content about 80%) 15 minutes.
2. Wash in water.
3. 0.1% aqu. sol. methylene azure (Azure A dye content 75–80%) 30 minutes.
4. Wash in water.
5. Differentiate in 95% alcohol with a few drops of xylol-colophonium, keeping slide in motion.
6. Absolute alcohol (several changes)
7. Xylol (several changes)
8. Xylol balsam.

From a personal communication from R. W. French it is learned that an essentially identical procedure may be used after formalin fixation, using eosin and azure C in place of the two dyes above specified.

AZURE B

Azure B is the tri-methyl derivative of thionin:



(A basic dye; absorption maximum about 652)

Like azure A, this compound was first recognized by Kehrmann (1906) as a constituent of polychrome methylene blue. It can easily be prepared in impure form by oxidation of methylene blue, and is now available from the National Aniline and Chemical Co., if anyone desires to obtain it.

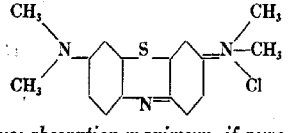
MacNeal (1925) states that its staining effects are much the same as can be obtained with a mixture of azure A and methylene blue, and that it therefore can be of little importance in polychrome methylene blue. Altho he adds that future study may change his opinion, his statement has generally been interpreted as meaning that azure B is not a satisfactory stain. Holmes and French (1926) also state that this dye has little staining value; but their conclusions are based upon work with an impure sample, the performance of which was so poor as not apparently to justify further purification. Miss Haynes' recent work (1927) on sections of fixed tissue, with this sample of azure B, agrees partially with this, altho she finds the dye, if used in buffered solution, rather better than fairly pure methylene blue in fresh solution. There are indications that a purer sample of azure B may be still better.

METHYLENE BLUE C. I. NO. 922

Synonym: *Swiss blue.*

Various grades denoted: *Methylene blue BX, B, BG, BB;* grade preferred for biological work: *Methylene blue Med. U. S. P.*

Methylene blue is theoretically tetra-methyl thionin:



(A basic dye; absorption maximum, if pure, about 667)
Solubility at 26°C: in water 3.55%; in alcohol 1.48%

The dye is so readily oxidized that it is practically impossible to obtain in pure form, the presence of some of the azures or of methylene violet being almost universal. Methylene blue has long been recognized as an important nuclear stain. It is notable, however, that all the staining solutions recommended for use on sections of fixed tissue call for some ripening or for the addition of alkali. Such solutions are bound to contain one or more of the azures. There are indications, in fact, that pure methylene blue is a relatively poor histological stain.

The methylene blue of commerce is generally a double salt, the chloride of zinc and methylene blue. The zinc is toxic, however; so for some time the zinc-free methylene blue chloride has been prescribed for medicinal purposes; hence the meaning of the term Methylene blue Med. U. S. P. The zinc double salt is less soluble, particularly in alcohol, so for most staining purposes is less desirable. The investigations of the Commission show that for all ordinary staining purposes the zinc-free compound is best; so that is the form at present recommended.

Methylene blue is perhaps the stain which the pathologist and bacteriologist would have the greatest difficulty in doing without, and it is of great value to the zoologist as well. It is employed for a greater variety of purposes than any other biological stain except possibly hematoxylin; and for this reason was the first dye to be given a thoro investigation by the Commission. It is used: first, as a nuclear stain in histology, for which purpose its strongly basic character as well as the ease with which it can be applied without over-staining, make it quite valuable; second, as a bacterial stain, notably in milk work and in the diagnosis of diphtheria, where it is especially useful because it has an affinity for the bacterial protoplasm as great as that of the rosanilin dyes, but is less intense, more selective in its action and more subject to differentiation; third in the vital staining of nervous tissue, where a non-toxic, basic dye is needed; fourth, in combination with eosin in the blood stains, thanks to the ease with which it can be partly converted into lower homologs such as the azures, and thus acquire polychrome properties; and lastly, as an indicator in the Levine eosin-methylene-blue medium for differentiating the colon and aerogenes organisms. There are indications that for the last mentioned purpose a methylene blue quite free from lower homologs is necessary.

It can be readily understood that an especially pure product is needed when the dye is to be used for vital staining or in blood work. For vital staining the U. S. P. zinc-free dye is always recommended, sometimes with even further purification; altho the investigations carried on by the Commission indicate that the U. S. P. product is sufficiently pure. For blood work some companies sell a "methylene blue rectified for blood stains." This grade,

however, is generally less pure than the medicinal or U. S. P. grade, and there seems no reason for specifying it. The same is true of various other grades such as those denoted BX, BG, etc., which are ordinarily purer than the textile dye, but less pure than the medicinal grade.

One serious bit of confusion has arisen from the designation "methylene blue for bacilli" which was used on a certain type of methylene blue imported before the war. This was the label placed on a certain type of zinc salt, containing a small amount of free chloride. Its designation seemed to imply that it was especially adapted for staining bacteria; recent investigations indicate that it should rather be considered not good enough for any other purpose! Even for staining bacteria it is not especially satisfactory; for the most common methylene blue solution of the bacteriologist is the Loeffler formula in which a certain amount of saturated alcoholic solution is used as a stock. Now, since the zinc salt is nearly insoluble in alcohol, such a stock solution contains little but the free methylene blue chloride present. For such reasons as this American manufacturers of biological stains have wisely discontinued the sale of the zinc salt under the above designation. One American concern, however, still sells a "methylene blue for bacilli," but it is entirely different from the pre-war imported product of that name. It is apparently methylene blue chloride, less pure than the medicinal grade but entirely satisfactory for staining bacteria and for many other staining purposes. The Commission has been unable, however, to find that it has any special advantages over the medicinal product as a bacterial stain. It must be repeated that for all staining purposes, except possibly some lines of vital staining, medicinal methylene blue is an eminently satisfactory grade.

Polychrome methylene blue. The oxidation of methylene blue into one or more of the compounds of lower methylation described on the preceding pages takes place in any methylene blue solution upon standing, especially rapidly if the solution be alkaline as in the Loeffler formula. All methylene blue solutions therefore, particularly if they have been standing any length of time, contain small amounts of the lower homologs, primarily azure A and azure B. These lower homologs are not only more violet in color than methylene blue itself but are more selective in their staining action. It is indeed claimed (Scott and French, 1924b) that the dye owes much of its value as a nuclear stain to the azures that are present, and that an extremely pure methylene blue is not so satisfactory for such use. A recent paper by Haynes (1927) indicates the correctness of such conclusions.

A methylene blue containing noticeably large proportions of the lower homologs is called *polychrome methylene blue.* The formation of these oxidation products may be hastened by boiling with alkali, as in Unna's formula.

Polychrome methylene blue is employed in many blood stains such as Leishmann's and Wright's; in the latter a methylene blue solution containing sodium bicarbonate is heated, and then eosin is added. Eosin enters into chemical combination with the basic dyes present, forming an insoluble compound which precipitates. This precipitate dissolved in methyl alcohol is Wright's stain. (For a more detailed discussion of the subject see Chapter VIII.)

Alcoholic-aqueous methylene blue:

Original statement of formula		*Emended statement*	
Conc. sol. methylene blue in alcohol	30 cc.	Methylene blue (90% dye content	0.3 g.
Distilled water	100 cc.	Ethyl alcohol	30 cc.
		Dissolve and mix with distilled water	100 cc.

Loeffler's alkaline methylene blue: Prepared like the above, except that instead of distilled water a 0.01% solution of KOH is employed to mix with the alcoholic solution of the dye in the above given proportions.

This formula was first employed when the methylene blues available were not so pure as today and were undoubtedly quite acid in reaction. (See Conn., 1929). It is not to be recommended, however, for the methylene blues now sold in America; they do not require neutralization and contain a sufficient proportion of the lower homologs (azures etc.) to give a good differential stain without treatment with alkali. The use of alkali with these methylene blues is likely to bring about too great oxidation; and accordingly they do not always have good keeping qualities when made up by the Loeffler formula.

Staining the diphtheria organism: Either of the above solutions (preferably the former, if Commission certified dye is employed) is often used for staining smears from throat cultures to determine whether the diphtheria organism is present. The technic employed is ordinarily very rapid staining followed by washing in water.

Carbol-methylene-blue: This may be made up like the first of the formulae above, using 5% aqueous phenol in place of distilled water. It is perhaps more often made up only 10 cc. of the alcoholic methylene blue to 100 cc. of 5% phenol. On the other hand, the following formula occurs which is even stronger than the Loeffler solution:

Carbol-methylene-blue (Kuehne):

(Formula quoted from Eyre, *2nd ed.*, 1915, p. 91)

Methylene blue	1.5 g.
Phenol	5.0 g.
Distilled water	100 cc.
Dissolve and mix with:	
Ethyl alcohol	10 cc.

In the source above cited, absolute alcohol is specified; but on account of the large amount of water called for, it is unnecessary, and 95% alcohol may be

substituted. This formula was based on pre-war methylene blue, having a dye content of perhaps 60%. The quantity called for should be reduced, therefore, when modern 90% dyes are employed; probably one of the other carbol-methylene-blue formulae given above is now to be preferred.

Unna's alkaline methylene blue solution:

(formula quoted from Mallory and Wright *8th ed.* p. 76)

Methylene blue*	1 g.
Potassium carbonate	1 g.
Water	100 c.c.

This solution should be diluted 1:5 or 1:10 for staining. If allowed to stand a few months at room temperatures this solution becomes polychromed. Its polychroming may be greatly hastened by the cautious use of heat. The ripened solution is known as Unna's polychrome methylene blue.

Goodpasture's acid polychrome methylene blue:

(formula quoted from Mallory and Wright *8th ed.* p. 76)

Methylene blue*	1 g.
Potassium carbonate	1 g.
Water	400 c.c.

Dissolve and boil for thirty minutes. When cool add 3 cc. of glacial acetic acid, and shake until the precipitated stain is redissolved. Boil for 5 minutes or until the solution is concentrated to a volume of 200 c.c. Upon cooling it is ready for use, and keeps indefinitely.

Terry's neutralized polychrome methylene blue (Terry, 1928). This is a modification of Goodpasture's formula, the chief difference being that an amount of acetic acid is added which will exactly neutralize the potassium carbonate. One determines by titration with phenolphthalein the quantity of a 12% potassium carbonate solution to neutralize 1 cc. of 10% acetic acid; to this amount of the carbonate solution is added sufficient 1% methylene blue (medicinal; about 90% dye content) to make a quantity of 100 cc. The alkaline solution is then heated by placing the container in a cold water which is brought to a boil. After 15 to 30 minutes from the commencement of boiling the staining solution is removed from the water bath, cooled slowly and neutralized by adding 1 cc. of 10% acetic acid. The author recommends dividing the alkaline solution into four portions of 25 cc. each before boiling and removing at the end of 12, 20, 25 and 30 minutes respectively; then each is neutralized separately by the addition of 0.25 cc. 10% acetic acid. In this way four solutions each polychromed to a different extent are obtained; and the one giving the desired effect may be selected.

Nissl's methylene-blue solution: The stain should be at least three to four months old, and shaken at the moment of filtering the quantity needed. It is prepared by carefully dissolving 1.75 g. of Castile soap in 1 litre of dis-

*Methylene blue of 90% dye content is undoubtedly satisfactory in these formulae without modification.

tilled water and adding to it 3.75 g. of methylene blue.* It is a good practice to shake the bottle vigorously from time to time, and to re-filter into the same bottle the amount of stain left in the watch-glass after staining one or more sections.

Held's methylene blue and erythrosin method:† For material fixed in alcohol, or preferably in picro-sulfuric acid, in van Gehuchten's mixture of alcohol chloroform and acetic acid, or in 1% $HgCl_2$ in 40% acetone. Embed in paraffin and fasten sections to slides by water method.

1. Stain with gentle heat for one or two minutes in:

Erythrosin (*B?*)	1 g.
Distilled water	150 cc.
Glacial acetic acid	2 drops

2. Wash with water.
3. Transfer slides to a mixture of equal parts of Nissl's methylene blue solution and 5% acetone; warm until all odor of the latter has disappeared.
4. Cool and differentiate in a 0.1% aqueous solution of alum until sections are reddish.
5. Rinse in distilled water.
6. Dehydrate as rapidly as possible in absolute alcohol.
7. Wash in xylol.
8. Mount in balsam.

The Breed method of counting bacteria in milk:

1. With a capillary pipette, take 0.01 cc. of the milk to be examined, place it on a microscopic slide and spread over an area of 1 sq. cm, with a stiff needle. The film may be made the desired size by placing the slide, during this operation, over a piece of paper or cardboard on which an exact square centimeter has been marked.
2. Dry with gentle heat on a level surface.
3. Dip in xylol a few minutes to dissolve out the fat.
4. Immerse in 90–95% ethyl alcohol a few minutes to fix the smear to the slide.
5. Place in alcoholic-aqueous methylene blue (see above) for 2 minutes. (Or carbol methylene blue may be used if a solution of better keeping qualities is desired.)
6. Wash briefly in 90–95% ethyl alcohol until the intense blue color changes to a faint tinge. This decolorizing may be omitted if the staining period has been made briefer (10–15 seconds), or if a more dilute staining fluid has been employed.
7. Dry and examine under microscope with an oil immersion objective.

If it is desired to count the bacteria, the eyepiece should contain a micrometer bearing a circle which has been calibrated so that the size of the field

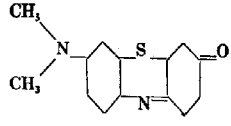
*No information available as to dye content of methylene blue for best results in this formula. As it is an old formula, the dye originally employed was probably not over 60–70% dye content, and the quantity called for should be decreased to obtain a solution comparable to the original.

†From Lee's Microtonist's Vademecum, 8 Ed. p. 415.

included with the microscope and lenses employed is exactly known. (For method of calibration see Breed and Brew 1916.)

METHYLENE VIOLET (Bernthsen)

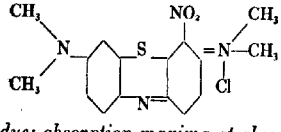
Methylene violet is formed whenever methylene blue is heated with a fixed alkali or alkali carbonate. It is a feeble base with the formula



Its preparation from methylene blue is more difficult than that of azure A. A fair yield (30 to 40 per cent) may be obtained by oxidizing methylene blue in dilute ammoniacal solution with potassium chromate and then driving off the ammonia by boiling with the addition of sodium carbonate. It may also be prepared from azure A by boiling this with dilute alkali carbonate. Methylene violet precipitates out as needle crystals, insoluble in water. It may be recrystallized from ethylene dichloride ($C_2H_4Cl_2$) in which it forms a deep carmin red solution. Altho insoluble in water when pure, methylene violet is soluble when mixed with methylene blue or with the azures. It plays an important part in the nuclear and granule staining of the polychrome methylene blue stains. A definite quantity of this dye is employed in the tetrachrome blood stain of MacNeal.

Methylene violet (Bernthsen 1885) is not a textile dye and must not be confused with methylene violet RRA or 3RA, which is C. I. No. 842.

METHYLENE GREEN C. I. NO. 924



(*A basic dye; absorption maxima at about 660, 607.*)
(*Solubility at 26°C: in water 1.46%; in alcohol 0.12%*)

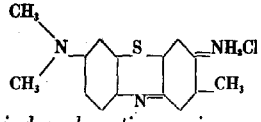
This dye is a mono-nitro methylene blue, obtained by the action of nitrous acid on methylene blue. The formula is probably as given above, but the exact position of the nitro group is uncertain.

It is occasionally used as a substitute for methyl green, especially by botanists in the case of wood and fixed chromatin, and gives good results in combination with eosin.

TOLUIDINE BLUE O C. I. NO. 925

Synonym: *Methylene blue O.*

This dye is closely related to thionin and to methylene blue in structure, and even more closely to methylene azure A:



(A basic dye; absorption maximum about 635.)
Solubility at 26°C: in water 3.82%; in alcohol 0.57%

Altho not a common textile dye, toluidine blue is more easily prepared than thionin or azure A—a fact of considerable importance, since in many procedures it may be substituted for one or the other of these dyes. It may be employed like azure A as a nuclear stain for sections of fixed tissue; and may be substituted for thionin in staining frozen sections of fresh tissue. It is a valuable general nuclear stain, being ordinarily employed in 0.3–1% aqueous solutions. It is widely employed in stains for Nissl granules and chromophylic bodies and has been proposed in a great variety of special procedures. Well known among the latter is Pappenheim's panchrome stain (of which it is an important ingredient) and the Albert stain which is at present widely replacing methylene blue in the diagnosis of diphtheria. The composition of the Albert stain follows.

Albert's diphtheria bacillus stain: Two solutions are used.

Solution No. 1 has the following formula:

Toluidine blue (80% dye content)	0.15	g.
Acetic acid (glacial)	1.	cc.
Alcohol (95%)	2.	cc.
Water (distilled)	100	cc.

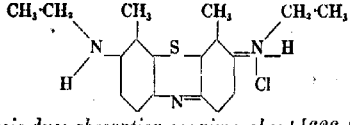
Solution No. 2 is the same as the iodine solution used in the Gram stain. The formula is:

Iodine	1.	g.
Potassium iodide	2.	g.
Water (distilled)	300	cc.

Smears are made on slides or cover glasses in the usual manner; fixed by heat and stained with the toluidine blue solution for 5 minutes. The stain is then drained off without washing and the iodine solution applied for one minute. It is then briefly washed with water and dried, preferably by means of filter paper. It is now ready for examination.

NEW METHYLENE BLUE N C. I. NO. 927

Synonym: *Methylene blue NN.*



(A basic dye; absorption maxima about [636.4], 588.)

Solubility at 26°C: in water 13.32%; in alcohol 1.65%

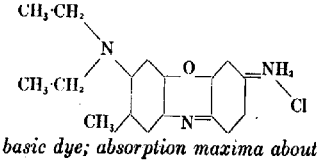
This dye has practically never been called for in microscopical work. The most interesting fact concerning it which has come to light relates to the Van Wijhe technic as applied by Louise Smith (1920) for staining the cartilage of frogs. The latter specified methylene blue, but the results could not be duplicated with any domestic or foreign methylene blue subsequently obtained. When furnished thru the Commission with samples of various stains to try, it was found that her earlier results could be duplicated with new methylene blue—a fact which not only implies mislabeling of her original supply of methylene blue, but suggests that new methylene blue may have some value in histological work.

3. THE OXAZINS

This group is like the thiazins in chemical formula except that the sulfur atom is replaced by an oxygen atom. Only a few of the dyes find use in microscopic technic, and they are not stains having very general appl cation.

BRILLIANT CRESYL BLUE C. I. NO. 877

Synonyms: *Cresyl blue 2RN* or *BBS. Brilliant blue C.*



(A basic dye; absorption maxima about 631.8 [579.5])

This dye is expensive to manufacture, and as it is not at present employed in any line of commercial dyeing, it must be prepared specially for the biologist. Its manufacturers, altho willing to coöperate to any reasonable extent, are naturally reluctant to carry on much experimental work with this dye unless they are sure the results will justify the outlay. This fact has caused some delay in bringing about its complete standardization.

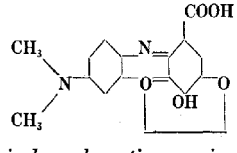
The dye has been employed to some extent in vital staining, Irwin (1927) for instance having found it the most favorable dye available for investigating penetration into living cells. Its best known use, however, is as a blood stain to bring out reticulated corpuscles by the technic of Robertson, and platelets by the method of Buckman and Hallisey (1921).

It is still uncertain just how satisfactory the American samples of this dye are for vital staining; but for the Robertson technic certain samples submitted have proved entirely satisfactory. There have been decided variations in this respect between the different samples and it has not yet proved possible to correlate them with any differences in chemical composition. The stain cannot, therefore, be regarded as standardized in a scientific sense. It can merely be said that the batches most recently submitted to the Commission and now on the market under the Commission certification, have been found satisfactory in blood work. There may still be room for improvement; if so, criticism from users of this stain will be greatly appreciated.

Robertson's method of counting reticulated blood corpuscles: A saturated solution of brilliant cresyl blue is made up in normal salt solution. This is kept as a stock solution. When a count is to be made, a small quantity of it is diluted 80 to 180 times* with normal salt solution and mixed with blood in a pipette for counting white cells in the proportion of one part of blood to twenty parts of brilliant cresyl blue solution. The mixture is shaken in the pipette for 5 minutes. The cells are thus equally distributed as well as stained. They are counted at once in fresh preparations, which are sealed with vaseline to prevent disturbances due to drying. At least 1,000 red corpuscles are counted at each test.

GALLOCYANIN C. I. NO. 883

Synonyms: *Alizarin blue RBN. Chrome blue GCB. Fast violet*

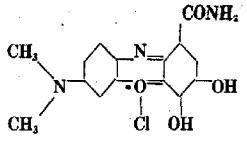


(A basic dye; absorption maximum about 636)

Proescher and Arkush find that the iron lake of this dye, prepared by boiling 2–3 minutes in a 5% aqueous solution of ferric ammonium sulfate, is a splendid nuclear stain and can be employed as a substitute for hematoxylin.

*The amount of dilution varies with the lot of dye employed and must be determined by a test in the staining technic in which it is to be used.

GALLAMIN BLUE C. I. NO. 894

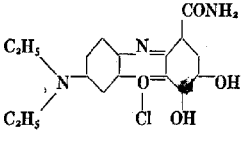


(*A basic dye; absorption maximum about 651*)

Employed in the form of its iron lake by Proescher and Arkush as a nuclear stain; the lake is prepared as described for gallocyanin above.

CELESTIN BLUE B C. I. NO. 900

Synonym: *Coreine 2R.*

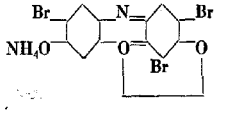


(*A basic dye; absorption maxima about 654.5 [600]*)

Preferred by Proescher and Arkush to either gallocyanin or gallamin blue as a nuclear stain. As in the case of these two other dyes, the iron lake, prepared by boiling 2–3 minutes in a 5% aqueous solution of ferric ammonium sulfate, is employed for the staining solution.

RESORCIN BLUE C. I. NO. 908

Synonyms: *Fluorescent blue. Iris blue.* Often called *Lacmoid.*

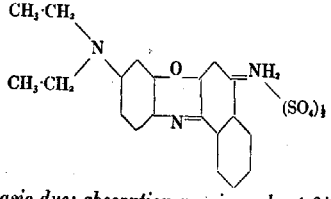


(*A basic dye; absorption maxima about [603, 588], 504*)

The name **lacmoid**, often applied to this dye, is more correctly given to its unbrominated derivative, which has been used for its indicator properties. Resorcin blue is employed by Tsvett as a microchemical reagent for the detection of callose.

NILE BLUE SULFATE C. I. NO. 913

Synonym: *Nile blue A.*



(*A basic dye; absorption maxima about 644.5, [592.2]*)

The use for which this dye is best known to the biologist is the Lorrain Smith fat stain. In this procedure the dye is boiled with dilute sulfuric acid, and thus hydrolyzed, with the introduction of oxygen in the place of the radical $NH_2(SO_4)\frac{1}{2}$, in other words producing a new dye of the class known as oxazones. This oxazone dye is red, and is fat-soluble. Nile blue sulfate itself, on the other hand, is not fat-soluble but combines readily with fatty acids. As a result the technic serves to distinguish between the free fatty acids in histological material and the neutralized fats, the former staining blue, the latter red.

Nile blue sulfate is used unaltered for staining living tadpoles previous to making transplants, in order to distinguish the grafts, also as a supravital stain for embryos (Detwiler) and as a vital stain for hydrae (Weimer).

Smith and Mair's stain for fat: Make a saturated aqueous solution of nile blue sulfate and add 0.5% of sulfuric acid. Boil under a condenser for an hour or two. Test the solution by shaking a little of it up in a test-tube with xylol; if a sufficient amount of the red oxazone has been formed the xylol will assume an intensely fluorescent red color. The sulfuric acid may be neutralized by adding an equivalent amount of caustic soda or the stain may be used in the acid condition. Sections cut on the freezing microtome are stained overnight at room temperature, or for a shorter period at 37°C. They are then differentiated in 2% acetic acid, washed in water and mounted in Farrants' medium or in gum. A good method for permanent preparations is to cover the section with a drop of gum acacia, allow this to become completely dry and then cover with balsam and a cover glass.

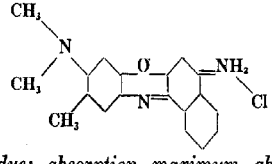
CRESYL VIOLET

Synonyms: *Cresylecht violet* (i. e. *cresyl fast violet*)

The exact chemical nature of imported cresyl violet is not yet known. It was stated in the previous edition of this book (p. 52) that the pre-war cresyl violet was a mixture of two dyes, one redder than the other. This impression was obtained from the

spectrophotometric study of the samples; and there is now reason to believe that it was not entirely correct. Altho each individual sample of the imported cresyl violet is probably a pure dye, the various samples examined frequently differ from one another and seem to be several closely related dyes.

At the present time a product of this name put on the market by the National Aniline and Chemical Co. seems to be a still different dye. This National Aniline cresyl violet is better known chemically than the imported product and is considered to have the formula:



(A basic dye; absorption maximum about 585)
Solubility at 26°: in water 0.38%; in alcohol 0.25%.

Like brilliant cresyl blue, this dye is not employed on a commercial scale. Its chief value in histology is on account of its metachromatic properties. It has been employed in making permanent preparations of nervous tissue, and is excellent for staining fresh areolar connective tissue, as it brings out various histological elements. According to Ehrlich (1910, II, p. 78) it stains nuclei violet, plasma blue, amyloid, mucin and mast cell granules red. Spiridonovitch employs it in the vital staining of white blood cells. Williams uses it for staining sections of fresh tumor tissue in biopsy work. It is also employed for making permanent preparations of fixed tumor tissue.

There is evidence that a stain satisfactory for one of these two latter purposes may not yield good results with the other. Williams, for example, finds rather better results with the National Aniline product in biopsy work, while from other quarters complaints have been received concerning the same batch of this dye when used for making preparations from fixed tumor tissue, for which the imported dye is very satisfactory.

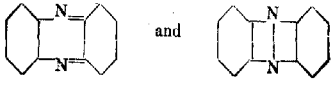
It is plain that further work is necessary both on the chemistry of the imported product and on comparative value of the two types in staining. Experimental work on this dye has not progressed fast, both because of the small number of biologists who employ it and because of the expense of manufacture; but it is hoped that eventually it will be as well standardized as the more commonly used stains.

Other oxazin dyes sometimes mentioned in connection with histology are:

Capri Blue. C. I. No. 876.
Naphthol Blue. C. I. No. 909. Synonym: *New blue B. Fast blue 3R. Phenylene blue. Meldola's blue. Indin blue 2RD.*

4. THE AZINS

The dyes of the azin group are derivatives of phenazin, $C_6H_4{\cdot}N_2{\cdot}C_6H_4$, a compound containing two benzene rings linked thru two nitrogen atoms in such a way as to form a third ring. Two formula are possible:

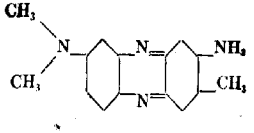


In the case of the first formula the quinoid ring is the chromophore; in the case of the second formula the azin group itself, $\begin{matrix}-N-\\ |\quad |\\ -N-\end{matrix}$, (see p. 22) is assumed to be the chromophore. The quinoid formula is generally preferred today.

Phenazin is weakly basic, but is not a dye as it does not contain auxochrome groups. In other words, it is a chromogen. Either an —OH group or one or more $—NH_2$ groups may be introduced to give it dye properties. The acids and bases are very weak if there is only one auxochrome group present, and their salts are readily decomposed. For this reason some of them are of use as indicators. Strong bases are encountered only among the safranins where basic character is derived not only from the two $—NH_2$ groups but also from one of the azin nitrogen atoms which becomes pentavalent and takes part in salt formation.

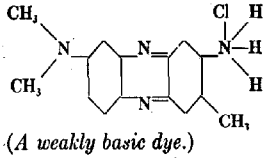
a. Amido-azins or Eurhodins

If one or more amino groups are introduced into a phenazin, a dye is formed of the class known as eurhodins. They are very weak bases, and therefore weak dyes; but as their salts are readily decomposed with a resulting color change, they form useful indicators. The best known of the group is toluylene red, base:



The chloride of toluylene red is the well known neutral red.

NEUTRAL RED C. I. NO. 825



(*A weakly basic dye.*)

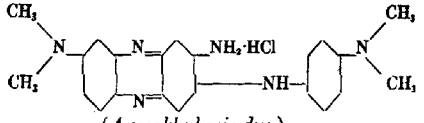
Solubility at 26°C: in water 5.64%; in alcohol 2.45%

This dye color is yellow in solutions a little below the neutral point (i.e., pH = 7.0) in reaction and red in weak acids, even the reaction of ordinary tap water being sufficient to bring out the acid color; at a higher range of acid it turns blue. This gives it some value as an indicator. As an indicator it is also used in bacteriological media for distinguishing the colon from the typhoid organisms, and for recognizing other forms; altho it is employed for this purpose much less today now that other dyes have been shown to have even greater value for the same type of work.

Neutral red is employed in a variety of staining methods. It is a weak nuclear stain, and has value for that purpose in certain procedures. Twort's stain for parasites in tissues is a neutral stain (see Chapter VIII) formed by combining neutral red with light green and dissolving the precipitate in alcohol. Neutral red finds use in the study of the Golgi apparatus in cells; but there is a dispute as to whether it is actually a specific stain for this structure or merely for the inclusions of the Golgi apparatus. Old ripened solutions are employed for bringing out the Nissl granules in nerve cells. It also has some use in general histological staining, particularly for embryological tissue in combination with Janus green, as recommended by Faris.

It has special value where a weakly basic, non-toxic dye is called for, as in vital staining. It is used for staining living protozoa, and as a vital stain for nuclei in tissue; also for the "vital" staining of blood, that is of fresh blood observed under a microscope in a moist chamber, and for staining fresh gonorrhoeal pus under similar conditions. It proves useful as an indicator of reaction of the contents of living plant cells. The chief draw-back to neutral red in vital staining is the toxicity of certain lots that have been on the market. This toxicity seems to be due to impurities present in the dye. Philips and Cohen (1927) have recently shown that these impurities can be eliminated more readily by preparing the dye as an iodide instead of a chloride. Their process, however, has not been adopted by manufacturers as yet, because of technical difficulties. Nevertheless the recent samples of neutral red chloride submitted for certification have been so well purified that they seem to be free from toxicity.

NEUTRAL VIOLET C. I. NO. 826



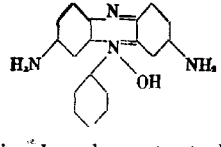
(*A weakly basic dye.*)

Solubility at 26°C: in water 3.27%; in alcohol 2.22%

This dye is very similar in its properties to neutral red, except that, due to its greater molecular weight, it is more bluish, giving a violet instead of a red color. It can be used as an indicator, but has been seldom used in histology. Unna (1921) however, has recently used it in a dye mixture employed in the study of chromolysis.

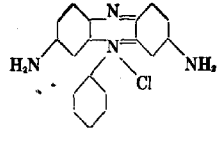
b. Safranins

Quite a long series of azin dyes are known in which one of the nitrogen atoms of the azin group is pentavalent and another benzene ring is attached to it. This pentavalent nitrogen allows the compounds to behave like ammonium bases; so with the amino groups which are always present, the basic properties of these dyes are very strong. The theoretical base of the simplest safranin would have the formula:



This form of ammonium base does not actually exist, as the safranin bases really occur in the form of anhydrides; but salts of these ammonium bases are the commonly known dyes. The commercial dyes are ordinarily chlorides.

There are two groups of safranins: the benzo-safranins in which the azin group unites two benzene rings; and the naphtho-safranins in which it unites two naphthalene groups. The simplest safranin is pheno-safranin, which is the chloride of the theoretical base just given, namely:



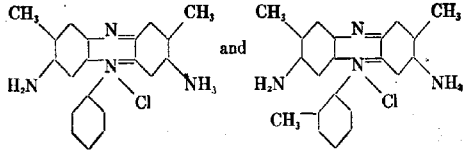
The commercial safranins are ordinarily methyl or ethyl substitution products of this; or occasionally phenyl substitution products. The one of greatest value to the biologist is generally called safranin O.

SAFRANIN O C. I. NO. 841

Slightly different shades: *Safranin AG, T, MP, Y,* and *G.* (Altho all included in C. I. No. 841 they are different from the grade here described.)

(A basic dye; absorption maximum about 515.)
Solubility at 26°C: in water 5.45%; in alcohol 3.41%

The common safranins of commerce, under various shade designations, are mixtures of di-methyl and tri-methyl phenosafranin:



The shade differs according to the proportion of these compounds present, the red being deeper according to the proportion of the tri-methyl compound in the mixture. The type safranin O, which proves best for ordinary biological purposes, can be defined as having its absorption maximum at 515mμ.

Safranin has always been a problem, because of its variability, especially in cytological work where it is often employed together with another basic dye, and a delicate balance between the two stains is necessary. The reason for this variation has been a puzzle ever since the Commission has begun its investigations of this dye. The question is not yet answered. It is realized at present, however, that among the safranin samples regarded as satisfactory there are two different types, a quick acting and a slow acting type, the former staining more intensely in a short period than the latter. This difference does not correlate with the dye content of the sample nor with the chemical nature of the dye itself so far as it is known at present. The reason for this difference is not yet understood; meanwhile one must vary the technic according to which type of safranin is employed. (See directions below under "Safranin with crystal violet in cytology.")

Grübler and Co. sell four types of safranin denoted: safranin pur, safranin gelb, safranin O wasserlöslich, and safranin spritlöslich. The first of these is a more violet dye, apparantly methylene

violet (C.I. No. 842). The others, to judge by samples purchased recently, are safranin O, and each is abundantly soluble either in alcohol or in water. The designations of the water and alcohol soluble types are therefore misleading; whether there was more difference between the two in pre-war days is a question still unanswered. At all events both types are still called for in some procedures; see quotation from Chamberlain under the directions for the Flemming triple stain, below. So far as known to the Commission, however, the two types are not necessary; and any satisfactory sample of safranin O can be employed for all desired purposes, provided the proper technic as regards time of staining and strength of solutions is used.

Safranin O is one of the most important nuclear stains known to the histologist. The botanist finds it especially valuable, as it brings out lignified and cutinized tissues in vascular plants, and can be employed in combination with a variety of contrast stains; it is valuable as a protein stain in plants, and can be used to stain spore coats. The cytologist makes use of it in the Benda technic to stain chromatin in combination with light green as a contrast stain; and even more widely in the Flemming triple stain, in which it is employed as a chromatin stain, together with gentian violet and orange G. The bacteriologist has some use for it, especially as a counterstain in the Gram technic (see p. 106).

The Flemming triple stain for mitosis; as modified by Chamberlain for use in plant cytology:*

Stains: "Make a 1% solution of alcoholic safranin in absolute or 95% alcohol, and after the safranin is completely dissolved, add an equal volume of a 1% solution of aqueous safranin in water, thus making a 1% solution of safranin in 50% alcohol. Use a 1% aqueous solution of gentian violet and a 1% aqueous solution of orange G." These directions, as above quoted from Chamberlain, are difficult to follow, chiefly because it is uncertain what is meant by aqueous safranin and alcoholic safranin. As no such distinction is recognized today, a 1% solution of safranin O (dye content about 90%) in 50% alcohol is to be recommended instead. For gentian violet employ crystal violet (about 90% dye content), unless the shade obtained is too blue to suit the user; in the latter case try methyl violet 2B (80–90% dye content). The orange G should be of 80–85% dye content.

Technic:

1. Paraffin sections, after the paraffin is dissolved out, are placed in 95% alcohol.
2. Stain in the safranin solution 3 to 24 hours.
3. Differentiate in 50% alcohol until stain is washed out of spindle and cytoplasm but not until the chromosomes begin to lose their bright red color. (If decolorization is too slow, 50% alcohol acidulated with one drop of concentrated HCl to 50 cc. may be applied for a few seconds, subsequently removing the acid by washing 15 to 30 seconds in plain 50% alcohol.)

*See Chamberlain's Methods in Plant Histology, 4 *Ed.* p. 64.

4. Dip 5 or 6 times in water.

5. Stain in the crystal violet solution 2 to 30 minutes, the time varying with the material being stained. The violet should stain the spindle, but not the chromosomes. For root tips Chamberlain recommends 2 to 10 minutes, for pollen mother cells, 5 to 10 minutes, for embryo cells of *Lilium* 10 minutes, while for germinating spores of liverwort, 30 minutes is not too long.

6. Dip 5 or 6 times in water.

7. Stain 30 to 60 seconds in the orange G solution.

8. Transfer briefly to 95% alcohol, then to absolute alcohol for 3 to 30 seconds.

9. Clear in clove oil.

10. Transfer to xylol.

11. Mount in balsam.

Safranin with crystal violet in cytology: These two dyes are often used together without orange G. According to Chamberlain one may in this case follow the above procedure but transfer from the violet directly to 95% alcohol and then proceed as directed. A method has recently been worked out in the Commission laboratory, however, which perhaps gives better results with the safranins now on the market. It must be recognized, as explained above, that there are two types of safranin O now available, the slow staining and the rapid staining type; a different procedure is required for each of the two types. The procedure given applies primarily to root tips fixed in Flemming, and embedded in paraffin.

1. Sections freed of paraffin are stained 15 minutes with a quick acting safranin or 8–10 hours with a slow acting sample, in a 1% aqueous solution of the dye.

2. Wash quickly with water.

3. Differentiate in 95% alcohol containing 0.2% light green SF yellowish (C. I. No. 670).

4. Remove green completely by very brief washing in water. Examine under microscope to see if safranin is removed from cytoplasm but is still deep in the chromatin; if not sufficiently removed from the former, differentiate and wash again as above.

5. Dip for a few seconds in 1% aqueous solution of crystal violet (dye content 90%).

6. Wash quickly in water.

7. Flood with absolute alcohol, then differentiate in clove oil until the crystal violet is removed from the cytoplasm but stains the spindle and the granules of the resting nucleus with the exception of the nucleoli.

8. Remove clove oil by flooding with xylol.

9. Mount in balsam.

Safranin with green counterstain: Safranin is often employed in cytology and histology without any other basic dye but with light green SF yellowish (C. I. No. 670) as a counterstain. This latter dye fades rapidly; and it proves advisable to substitute fast green FCF which is somewhat more permanent. The following technic for using safranin with the latter dye, as worked out

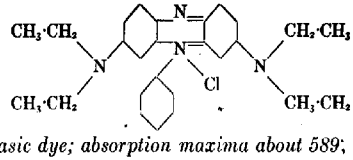
in the Commission laboratory, applies primarily to root tips fixed in Flemming's solution and embedded in paraffin:

1. Sections, freed of paraffin, are stained 15 minutes with a quick acting safranin or 8–10 hours with a slow acting sample, in a 1% aqueous solution of the dye. Safranin gives a more intense stain in a shorter time when dissolved in a 2.5% solution of sodium acetate.
2. Wash quickly in water.
3. Apply 0.5% solution of fast green FCF (85% dye content) in 95% alcohol until the safranin is extracted from the cytoplasm but remains bright red in the chromatin.
4. Flood with absolute alcohol; then xylol.
5. Mount in balsam.

AMETHYST VIOLET C. I. NO. 847

Synonyms: *Heliotrope B, Iris violet.*

This dye is tetra-ethyl pheno-safranin:



(*A basic dye; absorption maxima about 589*; [*545.5*])

Solubility at 26°C: in water 3.12%; in alcohol 3.66%

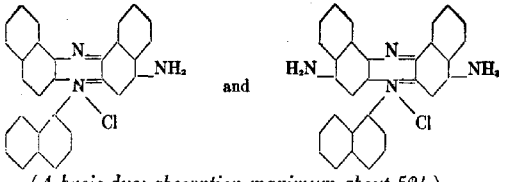
Amethyst violet has been used by Ehrlich and Lazarus as a basic dye in certain triple staining technics.

A further dye of this group which the biologist must take into account, altho it seems to have no significance as a stain, is methylene violet RRA or 3RA, C. I. No. 842 (syn.: *fuchsin* or *safranin extra blue*). This dye is a di-methyl safranin in which the methyl groups are introduced into one of the amino groups instead of directly into the benzene ring. It has no connection with the methylene violet of Bernthsen, which is one of the constituents of polychrome methylene blue; see p. 70. A recent sample has been found labelled "Safranin, pur", Grübler.

MAGDALA RED C. I. NO. 857

Synonyms: *Naphthaline red. Naphthaline pink. Naphthylamine pink. Sudan red.*

This is a naphtho-safranin, and is a mixture of the monamino and diamino compounds:



(*A basic dye; absorption maximum about 524.*)

A true magdala red put on the market before the war, and still sold by Grübler and Co., under the name of magdala rot *echt*, is quite expensive. This same company also sells a product "magdala rot der Handels," and a similar type has been sold by Coleman and Bell in this country. This so-called commercial magdala red is an entirely different dye, phloxine (see p. 130)—an acid instead of a basic dye, and one of an entirely different group. Chamberlain (1927) states that his work with magdala red was done with the latter type, hence actually with phloxine instead of the dye he thought he was using; his results cannot be duplicated with true magdala red. This means that in the well-known procedure for staining algae, using this dye in combination with anilin blue, one should actually employ phloxine, not magdala red. (See also discussion under phloxine, p. 130.)

A magdala red has been employed by Flemming as a nuclear stain, and by Kultschitzky for staining elastic tissue. No information is available, however, as to whether they used phloxine or true magdala red.

C. THE INDULINS

Indulins are similar to safranins but are more complex: being quite highly phenylated amino derivatives. The only one to concern us is:

NIGROSIN, WATER SOLUBLE C. I. NO. 865

Synonyms: *Nigrosin W, WL*, etc. *Gray R, B, BB. Silver gray. Steel gray. Indulin black.*

Nigrosin is not a pure dye, but is a mixture; and apparently the composition of different lots may vary. Ordinarily it is a mixture of a blue-black or violet indulin with a yellow dye in such a proportion that the resulting blend appears black. As the proportion of these two dyes is not always the same, different samples of nigrosin may vary in the amount of blue apparent to the eye. It is assumed that the biologist wants a nigrosin which appears distinctly black; this is certainly true in those procedures where it is used for a background stain. Accordingly the samples of nigrosin submitted which have a bluish hue have been refused certification by the Stain Commission.

Nigrosin is used in place of India ink as a background stain in the study of unstained bacteria; also as a stain for the background in contrast to fuchsin in the Dorner stain for bacterial spores. It is recommended by Ehrlich for staining the tissue of the central nervous system either alone or in combination with other stains, and by Jarotsky for staining pancreatic tissue following hematoxylin. Botanists use it in studying algae and fungi. Pfitzer's picronigrosin serves as a chromatin stain. Nigrosin is also used by Unna in combination with "orange" (orange G?) in the study of the process of chromolysis.

Dorner's spore stain:

1. Make a heavy suspension of the organism in 2 to 3 drops of distilled water in a small test tube.
2. Add an equal quantity of freshly filtered Ziehl's carbol fuchsin (formula on p. 96).
3. Allow the mixture to stand in a boiling water bath for 10–12 minutes.
4. On a cover slip or slide mix one loopful of the stained preparation with one loopful of a saturated aqueous solution of nigrosin.* (This solution must be filtered before use, and may be kept indefinitely if preserved with a few drops of formalin.)
5. Smear as thinly as possible and do not dry too slowly.

The above simple procedure can often be used when preparations are desired for examination only, and even backgrounds for exhibiting or photographing are not required. For the latter purposes, and especially in the case of slime-producing bacteria, the following p ocedure is recommended:

1. Make the suspension in 0.5 cc. bacteriological nutrient broth, or water.
2. Add 1 cc. of 10% gelatin solution.
3. Add 1 cc. of carbol fuchsin and stain as above.
4. Wash out the colloids with warm water, with the help of centrifuge or sedimentation.
5. Mix with nigrosin* and proceed as above.

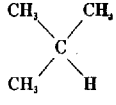
---

*Nigrosins, being mixtures, vary greatly in composition, and not all samples are equally satisfactory in this procedure. Certified nigrosin is always tested in this technic before it is approved.

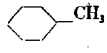
CHAPTER VI

THE PHENYL METHANE DYES

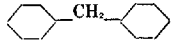
ONE of the most important groups of dyes, both from the standpoint of the dyer and from that of the biologist, is a group of substituted methanes, or in other words compounds with a central carbon atom. In methane, CH_4, it is possible to replace any of the hydrogen atoms with methyl, ethyl, or phenyl groups. If one H is replaced with CH_3, it becomes ethane, $CH_3 \cdot CH_3$. If two are replaced with CH_3 groups it becomes propane, $CH_3 \cdot CH_2 \cdot CH_3$; while if there are three substituent CH_3 groups it becomes iso-butane:



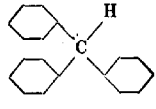
Similarly if one H is replaced with a phenyl group it becomes phenyl methane or toluene:



if with two it becomes di-phenyl methane:

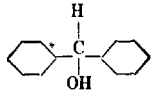


if with three it becomes tri-phenyl methane:



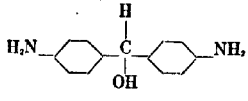
Certain substitution products of the di- and tri-phenyl methanes are among the most powerful dyes known.

Di- and tri-phenyl methane, themselves, are not dyes, nor are they chromogens. They lack both the chromophore and the auxochrome groups. The first step (theoretically) in converting them into dyes is to introduce an —OH group in the place of one of the unsubstituted H atoms of the methane nucleus. The compound thus formed, which bears the same relation to the phenyl methane as alcohol does to methane, is called a carbinol. A carbinol is methyl alcohol in which one or more of the hydrogen atoms may have been replaced with an alkyl radical or a benzene ring. Thus:

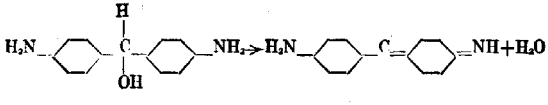


diphenyl carbinol

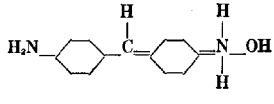
It is next theoretically possible to attach amino groups to the benzene rings. Thus in the case of di-phenyl carbinol it is possible to obtain di-amino di-phenyl carbinol:



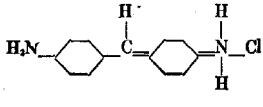
Now this latter compound contains the necessary auxochrome groups; but it is not yet a dye. No carbinol is a dye, because it lacks a chromophore group. The carbinols are important in dye chemistry, however, because upon dehydration a rearrangement of the bonds in the molecule takes place giving the quinoid benzene ring, which as we have seen is a powerful chromophore. Thus:



Now this latter compound is the anhydride of a true dye base. Upon hydration it should theoretically become:



Such a compound could exist only in watery solution. It is known only by its salts, the true dyes, as:



Altho the theoretical compound given above is the true dye base, the carbinols are often known as carbinol bases of the phenyl methane dyes or are sometimes called leuco-bases or color bases. They are not bases in the chemical sense, however, as they do not

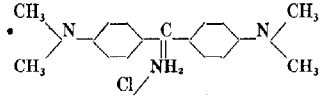
have basic properties. As stated above, they lack the chromophore group, and hence are colorless.

1. DI-PHENYL METHANE DERIVATIVES

The di-phenyl methanes are of practically no biological significance. Only one deserves mention here.

AURAMIN C. I. NO. 655

Synonyms: *Canary yellow. Pyoktaninum aureum. Pyoktanin yellow.*

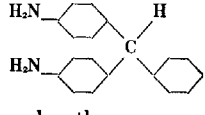


Altho of some use as a drug, auramin has little value in microscopic technic. It has been used by Fischel, however, in the vital staining of salamander larvae, and by Vinassa for staining plant sections.*

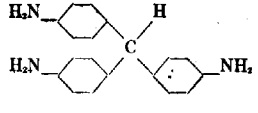
2. TRI-PHENYL METHANE DERIVATIVES

There are two groups of tri-phenyl methanes to concern us, the amino and the hydroxy derivatives. The former, which are much the more numerous, are very strongly basic, thanks to the amino groups, unless sulfonated like light green or acid fuchsin. The rosolic acid dyes, on the other hand, are hydroxy phenyl methanes, the amino groups being replaced by hydroxyl groups; they are therefore acid instead of basic dyes.

There are likewise two subdivisions of the amino derivatives, the di-amino tri-phenyl methanes and the tri-amino tri-phenyl methanes. These two groups are derivatives respectively of: di-amino tri-phenyl methane



and tri-amino tri-phenyl methane, or pararosanilin.



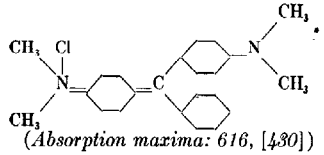
*Literature references to the procedures mentioned in this chapter may be found on pp. 174 to 195 and 204 to 216.

The individual dyes of this series are substitution products of these two compounds and differ from one another in the number of methyl, ethyl, or phenyl groups introduced, and according to whether they are introduced into the amino groups or directly onto the benzene rings.

a. Di-amino Tri-phenyl Methanes

MALACHITE GREEN C. I. NO. 657

Synonyms: *Emerald green. New victoria green. Diamond green. Solid green. Light green N.*



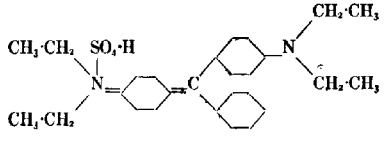
(*Absorption maxima: 616, [430]*)

Malachite green is a rather weakly basic dye that has been used in the past for various histological purposes; as by V. Beneden for staining Ascaris eggs, by Petroff for staining erythrocytes, and by Maas as a contrast stain following borax carmin. Today it has very largely been replaced by methyl green; but it is now often used by botanists for staining host tissue in plants infected with fungi, according to the technic of Pianese (with acid fuchsin and martius yellow), which was originally applied to cancer tissue. (See under martius yellow, p. 41).

BRILLIANT GREEN C. I. NO. 662

Synonyms: *Ethyl green. Malachite green G.*

This is a basic dye which is generally known in the form of the sulfate:



(*Absorption maximum: 623.*)

Brilliant green is not used, to any great extent at least, as a stain, but finds frequent employment as a constituent of bacteriological media. It is used for at least three different purposes: (1) as a constituent of Krumwiede's brilliant green bile media for plating

water in order to distinguish the colon organism from other lactose fermenting organisms; (2) it is employed in media used in searching for the typhoid organism in stools, where its value comes from its ability to inhibit the colon organism; (3) it is used as a constituent of enrichment media for the stimulation of the typhoid organism, in which it must prevent the growth of the normal colon types but not be toxic to the typhoid organism.

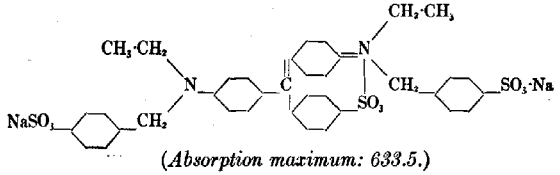
It has proved much more simple to find a brilliant green satisfactory for colon diagnosis in water work than for the isolation and enrichment of the typhoid organism. A recent paper by Rakieten and Rettger (1927) has shown the difficulty encountered in this latter instance. From their paper, however, it will be noticed that one satisfactory American sample of brilliant green was found by them. Since the writing of the paper in question these same authors have obtained equally satisfactory results with other samples. In fact all samples of this dye submitted for certification are tested by this technic, usually by one of the authors of this paper, before certification is granted.

In this way the supply of brilliant green on the American market is now being quite well controled; altho the problem presented by this dye is not actually solved, as it is not known why some samples are satisfactory for this purpose and others unsatisfactory.

LIGHT GREEN SF YELLOWISH C. I. NO. 670

Synonyms: *Light green 2G, 3G, 4G,* or *2GN. Acid green* (with various shade designations). *Fast acid green N.*

This is a derivative of brilliant green, which is sulfonated and is therefore an acid dye.



(*Absorption maximum: 633.5.*)

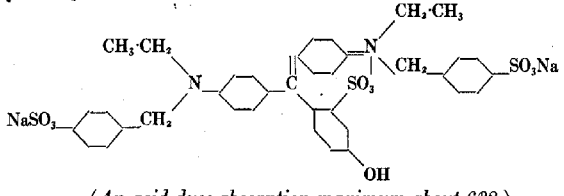
Solubility at 26°C: in water 20.35%; in alcohol 0.82%.

Light green is a valuable plasma stain often used for staining tissues in contrast to iron hematoxylin, altho it fades badly if exposed to bright light. It is a very valuable counterstain to safranin, especially after Flemming fixation, thus finding employment in cytological work. It photographs well. It is a constituent, together with neutral red, of the compound dye employed in the Twort stain for microörganisms in tissues. In plant histology it is a useful cytoplasm and cellulose stain. Its greatest drawback is that it fades rapidly and is therefore not very

permanent. Where greater permanency is desired the following dye may often be substituted for it.

FAST GREEN FCF

This is a dye, very closely related to light green SF yellowish, which has recently been proposed by Johnson and Staub (1927) as a food dye.



(An acid dye; absorption maximum about 628.)

Solubility at 26°C: in water 16.04%; in alcohol 0.35%

This dye has been tried in the Commission laboratory as a substitute for light green SF yellowish (see Haynes 1928). It has been found to give staining effects very much like the latter and to be considerably less subject to fading. Slides have been exposed to direct sunlight for a few weeks and have still retained the green color. This dye is therefore to be recommended for such use.

b. Tri-amino Tri-phenyl Methanes (Rosanilins).

The simplest rosanilins are the dyes sold as basic fuchsin. This term seems to be somewhat loosely used to apply to two or three different dyes and to various mixtures of them. The dyes known as fuchsin differ from the methyl violets and other rosanilins in that the amino groups are not methylated or substituted in any other way. The fuchsins may, however, have methyl groups introduced directly onto the benzene rings instead of into the amino groups; and the different fuchsins vary from one another in the number of such methyl groups present. There are four primary compounds theoretically possible, namely with no methyl group, and with one, two, and three substituent methyl groups respectively.

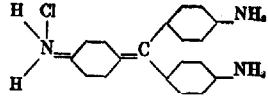
BASIC FUCHSIN

Synonyms: *Diamond fuchsin. Magenta. Rubin. Anilin red.*

The dyes ordinarily known to commerce as basic fuchsin are represented by C. I. No. 677, which is a mixture of pararosanilin and rosanilin. Both of these are obtainable in fairly pure form, as is also the compound with three substituent methyl groups, namely new fuchsin. The formulae of these three compounds follow:

Pararosanilin C. I. NO. 676

Synonyms: *Basic rubin. Para-fuchsin. Para-magenta.*
This compound is triamino-triphenyl chloride.

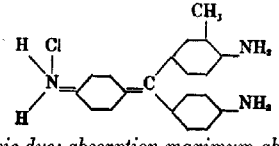


(A basic dye; absorption maximum about 539.)
Solubility at 26°C: in water 0.26%; in alcohol 5.93%

This dye is the chief constituent of the majority of samples of basic fuchsin submitted for certification as biological stains. It is sometimes furnished as the hydrochloride, sometimes as the acetate, generally the latter.

Rosanilin

This compound is mono-methyl fuchsin, or triamino-tolyl-diphenyl-methane chloride.



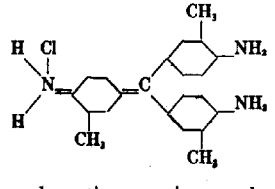
(A basic dye; absorption maximum about 542.)
Solubility at 26°: in water 0.39%; in alcohol 8.16%

It is not a textile dye, and is not found free from pararosanilin unless specially prepared.

New fuchsin C. I. NO. 678

Synonyms: *Isorubin. Fuchsin NB.*

This compound is tri-methyl fuchsin, or triamino-tritolyl-methane chloride:



(A basic dye; absorption maximum about 544, 545.)
Solubility at 26°C: in water 1.13%; in alcohol 3.20%

From the general rule concerning alkyl substitution it will be understood that new fuchsin is the deepest in shade of these three dyes, pararosanilin the least so. The commercial fuchsins, therefore, which consist of mixtures of rosanilin and pararosanilin vary in depth according to the proportion of rosanilin present.

It has proved from the investigations of the Stain Commission that not all basic fuchsins furnished as biological stains are mixtures of these two compounds. Many of the samples prove to be fairly pure pararosanilin; while others are deeper in shade than rosanilin and presumably contain appreciable quantities of new fuchsin. All of these dyes differ so very slightly from each other in shade that it takes very careful comparison to distinguish one from another, and for many purposes they may be used interchangeably.

The basic fuchsins are among the most powerful nuclear dyes, and find many biological uses. They are valuable stains for mucin, for elastic tissue, and for bringing out the so-called fuchsinophile granules. A basic fuchsin is often employed for staining the nuclear elements of the central nervous tissue. This group of dyes is almost indispensable in bacteriology, particularly in the Ziehl-Neelson method, with its various modifications, for differentiating the tubercle organism and thus making possible the diagnosis of tuberculosis. One of the basic fuchsins (usually a pararosanilin) is used as an indicator to distinguish the typhoid organism from other closely related forms, by means of the Endo medium in which it is changed to a colorless compound by the action of sodium sulfite. This medium (which contains lactose) remains colorless in the presence of the typhoid organism, which does not attack lactose; but becomes colored in the presence of organisms like *Bacterium coli* which ferment the lactose.

This Endo reaction is very similar to, if not identical with, the behavior of basic fuchsin in Schiff's reagent for detection of aldehyde. In this reagent basic fuchsin, decolorized with sulfurous acid, is similarly restored to its original color by the presence of any aldehyde. This reaction is important to the biologist not only because of its relation to the Endo medium, but because Feulgen and his associates (Feulgen and Rossenbeck, 1924; Folgen and Voit, 1924a) have employed this dye as a microchemical reagent to distinguish thymonucleic acid from the nucleic acids containing pentoses. This depends upon the fact that if one submits the nucleic acids to a mild partial hydrolysis, there is a liberation of aldehyde groups in the case of thymonucleic acid which restore the color of the fuchsin. This is called the "nucleal reaction" by Feulgen, the ending "-al" indicating the aldehyde-like nature of the substance upon which the reaction depends. This reaction is coming to have value in cytology. Considerable importance has been attached to it lately, as cancer cells, under

certain conditions at least, have been shown (see, for instance, Cowdry, 1928) to give an unusually strong nucleal reaction. A similar reaction ("plasmal reaction") is employed by Feulgen and Voit (1924b) for demonstrating aldehyde in cytoplasm, which becomes violet instead of red with this fuchsin reagent.

Until recently little information was at hand to show which of these dyes was most satisfactory for any one of the various purposes for which basic fuchsin is specified. A report (Conn, 1926) more recent than the first edition of this book discussed the situation; but at present later information is available which can be summed up as follows.

For ordinary histological work it apparently makes little difference which of these basic fuchsins is employed, the only distinction being in the shade produced, which is a slightly yellower red with pararosanilin and slightly bluer red with new fuchsin than with rosanilin. Perhaps the choice between them is merely a matter of individual peculiarities in color perception. The same is true when the dye is used as a simple stain for pure cultures of bacteria. In the stain for the tubercle organism on the other hand, distinctly better results have been obtained with the higher members of the group than with pararosanilin. Splendid results have been obtained with new fuchsin; also with unknown members of this group, appearing on spectrophotometric analysis to be largely rosanilin or possibly to contain an appreciable amount of new fuchsin. The reason for the better results with these higher homologs is undoubtedly because of the necessity of sharp, deeply stained tubercle organisms to contrast with the other bacteria which take the counterstain.

One must not, however, be too hasty in drawing the conclusion that new fuchsin or rosanilin is necessary for such special staining reactions as these. Equally good results have been obtained with certain samples of pararosanilin. Now pararosanilin is generally furnished in the form of the acetate, and in the course of preparation a certain excess of sodium acetate is bound to result that is not entirely removed from the finished product. Abundant evidence is at hand that the staining action of a dye may be greatly influenced for better or worse (see Chap. II) by changing the amount of such a salt present. It is considered probable that the difference in the behavior of different rosanilin samples may be due to some such cause. For the present, therefore, no way is known of controling the situation except to test each lot submitted as to its behavior as a stain for the tubercle organism.

Even less is known at present as to why some samples are more satisfactory than others for use in the Endo medium. To anyone who has worked much with this medium, it is a matter of common observation that different lots of fuchsin do not de-

colorize equally readily with sodium sulfite. Some of those that do decolorize in an apparently normal manner show too much restoration of color on standing to be satisfactory in actual use. Others fail to show sufficient restoration of color when inoculated with the colon organism in the Endo medium, failing most frequently to develop the metallic sheen which should characterize this organism. It is still uncertain just which basic fuchsins give satisfactory results by this technic, and which do not. It has been found that new fuchsin is entirely unsatisfactory; so apparently is the compound intermediate between rosanilin and new fuchsin, namely triamino ditolyl methane chloride, which has been experimentally prepared at the Color Laboratory of the Department of Agriculture. Good results have been obtained with pararosanilin and rosanilin, also with an unknown type of basic fuchsin appearing on spectrophotometric analysis to contain a higher derivative than rosanilin. On the other hand, some pararosanilins and some rosanilins have been entirely unsatisfactory. No correlation moreover, has yet been observed with the amount or nature of mineral impurities present. In other words here again the tests must be of a purely empirical nature.

French (1926c) has suggested for this purpose a test to determine first the amount of sulfite required to decolorize a definitely known amount of the dye under investigation; and secondly, the amount of formaldehyde required to restore the color. This test, as already brought out (Conn 1926), correlates in general with the results in actual use, but it has been found since the publication of the article just mentioned that there are too many exceptions for the test to be absolutely reliable. The test, therefore, actually employed in the Commission laboratory in the case of samples submitted for certification is as follows:

> A saturated alcoholic solution of the sample is prepared, and is diluted in alcohol in various proportions: 1 to 1, 1 to 2 etc., up to 1 to 10. To 10 cc. lots of a 2.5% solution of sodium sulfite is added 0.5 cc. of each of these fuchsin solutions starting with the strongest until one is found which decolorizes promptly to a straw color. This decolorized solution together with the one containing the next larger and the one with the next smaller quantity of fuchsin are made up into separate lots of Endo agar by the formula given below. Plates are poured and streaked with pure cultures of members of the colon group of bacteria. After one day's incubation at 37°C. the plates are examined to observe whether the correct type of color restoration has occurred.

Very often a sample that is satisfactory for the Endo medium is unsatisfactory as a stain for the tubercle organism, and *vice versa*. As a result, one of the American stain companies has put on the market two products, one for staining, the other for use in the Endo medium. The most recent batches of American manufacture are, however, suitable for both of these procedures. In what

respect they differ chemically from those that are satisfactory for only one of the above mentioned purposes has not been determined.

Plainly the important point is that any basic fuchsin sold be labelled so that the user can tell for which of these purposes it is adapted. All fuchsins certified by the Commission are so marked, but it would also be desirable that the label also tell whether the dye is a pararosanilin, a rosanilin, or a mixture of the two, and whether it is furnished as a chloride or as an acetate. This is not so commonly done at the present time; and as a matter of fact, the stain company itself does not always have this information. New fuchsin, it will be remarked, is probably always correctly labelled.

Ziehl's carbol-fuchsin:

Old statement of formula		*Emended statement of formula*	
		Solution A	
Sat. Alc. Sol. basic fuchsin	10 cc.	Basic fuchsin (90% dye content)	0.3 g.
5% sol. carbolic acid	100 cc.	Ethyl alcohol (95%)	10 cc.
		Solution B	
		Phenol	5 g.
		Distilled water	95 cc.
		Mix Solutions A and B.	

Staining the tubercle organism: Various methods have been given for determining the acid-fast properties of an organism; but all are really variations of the same general procedure: staining deeply with carbol fuchsin, then decolorizing with acidified alcohol, followed or accompanied by a counterstain.

The Ziehl-Neelsen method* is: carbol fuchsin with gentle steaming for 3 to 5 minutes or cold for 15 minutes; wash in water; decolorize in 95% ethyl alcohol containing 3% by volume concentrated hydrochloric acid until only a suggestion of pink remains; wash in water; counterstain with saturated aqueous methylene blue or Loeffler's methylene blue; wash and dry.

The Ziehl-Gabbet method† is similar but calls for simultaneous decolorizing and counterstaining in 2% methylene blue in 25% sulfuric acid (sp. gr. 1.18). For Pappenheim's modification, see p. 115.

Mallory and Wright 1924) recommend the following: carbol fuchsin with gentle steaming for 1 to 4 minutes; wash in water; decolorize in 70% ethyl alcohol containing 1% by volume of concentrated hydrochloric acid until red disappears (not more than a few seconds); wash in water; wash in 95% alcohol for 30 seconds; wash in water; counterstain for 30 seconds with Loeffler's methylene blue solution; wash and dry.

The Spengler method‡ calls for: carbol fuchsin with gentle steaming for 3 to 5 minutes; without washing apply for 2 or 3 seconds a mixture containing equal parts of 96% ethyl alcohol with 5% aqueous picric acid the latter

*See Stitt, (1923) p. 58–9.
†See Med. War Manual No. 6 of U. S. Army 1918 p. 25.
‡From Mallory & Wright, 1924, p. 440.

having been allowed to stand 24 hours and then filtered before adding to the alcohol); apply 3 to 4 drops of 15% nitric acid; after 5 seconds pour off nitric acid, and apply picric-acid-alcohol again until yellowish; wash and dry.

The Frothingham method of staining Negri bodies:* A thin slice of the brain tissue (chiefly gray matter), cut at right angles to the surface, is placed about one inch from the end of a slide. It is spread out into a moderately thin layer by means of a cover glass placed on it and moved along the slide. The preparation is then partially air-dried and fixed in methyl alcohol for about five minutes.

Stain:	Sat. aqu. sol. methylene blue	4 drops
	Sat. alc. sol. basic fuchsin	6 drops
	Distilled water	30 cc.

The staining solution is made up fresh and applied briefly. To obtain satisfactory results it may be necessary to vary the proportion of the two dyes and to experiment with different periods of staining; no definite dye content has ever been specified as preferable in the case of either dye.

Formula of Endo medium: Dissolve 10 g. beef extract and 10 g. peptone in 1000 cc. distilled water; add 30 g. air dried agar and dissolve by autoclaving; add 10 g. lactose and distribute in flasks, 100 cc. to the flask. Sterilize. Just before use prepare 10 cc. of a 2.5% solution of sodium sulfite. Add to this 0.5 cc. of an alcoholic solution of basic fuchsin, (a lot certified for the Endo medium) which should ordinarily be between 1.5 and 0.6%, but varies with the lot of fuchsin used. The exact strength to employ may be learned by preparing four different concentrations such as 1.5, 1.0, 0.75 and 0.6%, and using the most concentrated one which becomes reduced to a light straw color upon addition to the sulfite in the proportions given. Add the 10.5 cc. of this decolorized solution to 100 cc. of the medium just before pouring into the plates.

ACID FUCHSIN C. I. NO. 692

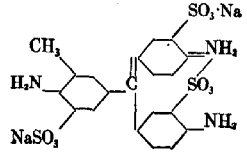
Synonyms: *Fuchsin S, SN, SS, ST,* or *S III. Acid magenta. Acid rubin.*

(*An acid dye; absorption maximum about 545.*)

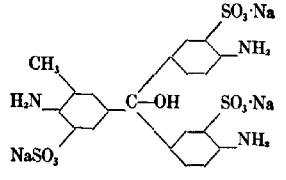
This dye owes its acid character to the fact that it is a sulfonated derivative of basic fuchsin. Acid fuchsins are ordinarily rather complex mixtures. As there are four primary basic fuchsins possible, according to the degree of methyl substitution, and as each may yield at least three different compounds on sulfonation, fully a dozen acid fuchsins are theoretically possible, and samples are hardly to be expected which are not mixtures of several.

The generally accepted formula of one of the homologs present in acid fuchsin, namely the di-sodium salt of rosanilin trisulphonic acid, is:

*From Mallory & Wright, 1924, p. 440.



The bond connecting one of the sulfonic groups with an amino group attached to a different benzene ring is assumed to exist in order to account for the fact that altho only two of the sulfonic groups are neutralized with sodium, the compound acts as tho it has no free acid. In other words, it is a case of intramolecular salt formation. Now when the tri-sodium salt is formed, this bond is broken down, whereupon the quinoid ring disappears and the following compound is produced:



This compound, it will be seen, is a carbinol in structure, and as it lacks the quinoid ring it is colorless; but it is very readily converted into the di-sodium salt by the addition of acid, whereupon the color again appears. This property makes acid fuchsin of use as an indicator. The decolorized solution of acid fuchsin neutralized with sodium hydrate is called the Andrade indicator. It is used quite extensively in bacteriological work, because of the striking reaction when its color is restored by acid-forming bacteria. As an indicator to show hydrogen-ion concentration at all accurately, however, it is found to have much less value than the phthalein and sulphonphthalein dyes (see pp. 133 to 140).

Acid fuchsin is a widely used plasma stain, which has also been recommended for a number of special uses. Among the best known are: the Van Gieson connective tissue stain, in which it is used with picric acid after hematoxylin to differentiate smooth muscle from connective tissue (see p. 40.); the Ehrlich-Biondi stain, in which with methyl green and orange G it is employed in histology and for staining blood smears; and the Ehrlich tri-acid stain for blood, which is a "neutral" combination with orange G and methyl green. In plant histology it is used to stain the cortex, pith and cellulose walls; while the Pianese stain (with malachite green and martius yellow; see p. 41), originally applied to cancer tissue, is now used by plant pathologists in studying infected vas-

cular plants. It is used with methyl green, by Altmann, Bensley and Cowdry as a stain for mitochondria. To the pathologist it is quite valuable as a constituent (with anilin blue and orange G) of the Mallory connective tissue stain. (See p. 114.)

Recent work by Scanlan, Holmes, and French (1927) has shown that many lots of acid fuchsin are unsatisfactory because of the rapidity with which they fade in Van Gieson preparations. These writers find that this fault arises from lack of control of the process of sulfonation. They show that if this process is carefully controlled by the use of a constant temperature and a definite period of time, a uniform product may be obtained. By the method which they propose in this article an acid fuchsin has been prepared on a small scale which is free from the fault above mentioned; it not only gives permanent Van Gieson preparations, but proves much more satisfactory—and for the same reasons—in the Mallory connective tissue stain.

Bensley's method for staining mitochondria.*

1. Fix tissues 24 hours in the following: 2% osmic acid, 2 cc.; 2.5% potassium bichromate, 8 cc.; glacial acetic acid, 1 drop.
2. Wash and embed in paraffin by usual steps.
3. Mount by the water method, remove paraffin with toluol, then pass thru absolute alcohol to water.
4. Treat for from 30 seconds to one minute (determined by trial) with a 1% solution of potassium permanganate; then for the same length of time with a 5% solution of oxalic acid. The permanganate extracts the mordanting elements of fixation and the oxalic acid removes the permanganate.
5. Wash thoroly in water.
6. Stain 5 minutes in Altmann's acid fuchsin (acid fuchsin† 20g., anilin water 100 cc.) which has previously been warmed to 60°C.
7. Wash thoroly in water.
8. Dip for an instant into a 1% solution of methyl green.†
9. Wash rapidly, dehydrate in absolute alcohol (avoiding alcohols of intermediate strengths), clear in toluol and mount in balsam.

Result: mitochondria, red; chromatin, green.

Toluidine blue may be substituted for methyl green in this technic. If the material does not stain well with the acid fuchsin, or if the methyl green or toluidine blue obliterates it, treat the sections with a 2.5% aqueous solution of potassium bichromate for about half a minute, and rinse in water just before staining in acid fuchsin.

---

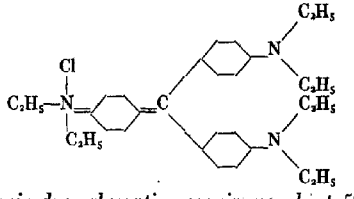
*From Guyer, 1917, p. 145.

†No information is available as to the most satisfactory types of these dyes to employ or of the dye content necessary to give good results. Commerical samples of both stains vary considerably, and it is possible that the irregularity referred to in the last paragraph may be due to this cause.

ETHYL VIOLET C. I. NO. 682

Synonym: *Ethyl purple 6B.*

Ethyl violet is hexa-ethyl pararosanilin having the following formula:



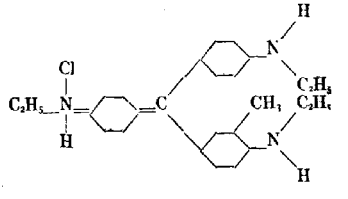
(*A basic dye; absorption maximum about 596*)

It has been called for by Bowie (1924) in a neutral stain combination with Biebrich scarlet to stain the islets of Langerhans.

HOFFMAN VIOLET C. I. NO. 679

Synonyms: *Dahlia. Iodine violet. Red violet. Violet R, RR* or *4RN. Primula R.*

These various names are applied in a somewhat indiscriminate way to dyes intermediate in shade between basic fuchsin and methyl violet. Theoretically they are mixtures of methylated and ethylated pararosanilins and rosanilins, having fewer than five ethyl or methyl groups. Of these compounds, the formula for tri-ethyl rosanilin is:



(*A basic dye.*)

In actual practice, however, the names applied to this group of dyes are very loosely employed, and one is often furnished under such a label as dahlia or Hoffman violet a mixture of basic fuchsin and methyl violet, having a shade about the same as one of the compounds just mentioned. Two samples of "dahlia" that have recently been called to the attention of the Commission differed very greatly, one being nearly as red as fuchsin, the other nearly as blue as methyl violet. There was every reason to believe that one was a fuchsin with a small addition of methyl violet, the other a methyl violet with a little fuchsin added. The latter

dyes are much more cheaply prepared than the true Hoffman violets, and the substitution is natural considering that the shade obtained can be the same.

Hoffman violet has been called for by Ehrlich and by Unna for staining mast cells; by Juergens for staining amyloid, which it colors red, while the cytoplasm is colored blue. Now it is possible that for one of these procedures or for some other similar one, a true Hoffman violet is necessary; on the other hand the shade obtained may be the important matter, in which case as satisfactory results should be obtainable with a mixture of fuchsin and methyl violet. This is a matter that should be further investigated; for if the cheaper fuchsins and methyl violets can be substituted for the Hoffman violets, the substitution can be made by the biologist himself. It is interesting to remark that one supply house not long ago sent out a bottle of crystal violet, unintentionally mislabeled dahlia, to a biologist ordering the latter dye. This biologist shortly wrote to the company that he would like more of it, as it was the best lot of dahlia he had ever used; in looking the matter up to duplicate the former order, the company discovered its mistake.

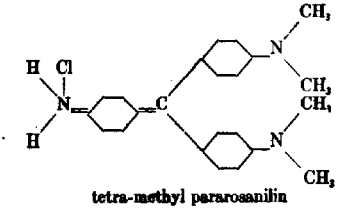
It is extremely difficult to determine just what dye in this group is referred to by any given name. Thus a rosanilin violet has been mentioned as having been employed in a stain for Negri bodies; and it is uncertain whether it is a dye of the above group or of the group following. Any biologist employing one of the violet rosanilins should be especially careful in publishing his work to give all the information furnished on the label as to manufacturer and the nature of the dye; and should preferably verify all such work, before publication, by using a dye of known composition.

METHYL VIOLET C. I. NO. 680

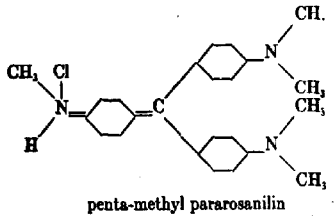
Synonyms: *Dahlia B. Paris violet. Pyoktanin blue. Gentian violet.*

Various shades denoted: Methyl violet 3R, 2R, R, B, 2B, 3B, BBN, BO, 3V.

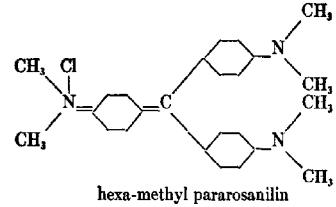
The various dyes denoted methyl violet are mixtures of tetra-, penta-, and hexa-methyl pararosanilin:



tetra-methyl pararosanilin



penta-methyl pararosanilin



hexa-methyl pararosanilin
(crystal violet)

(Basic dyes; absorption maxima: 583-584 in 90% alcohol.)

Solubility at 26°C: in water 2.93%; in alcohol 15.21%.

In the case of these compounds, as in the case of other series of homologs differing in extent of methylation, the shade is deepened by the introduction of each methyl group. Hence the various mixtures known to the trade as methyl violet vary from reddish to bluish violets according to the relative amounts of the more and less completely methylated compounds present in the mixture. This is the significance of the various shade designations listed above, R's indicating the reddish shades, and B's the bluish shades. Of these various shades the bluer ones seem to be best for biological purposes, methyl violet 2B having been found satisfactory for practically all purposes for which methyl or gentian violet is ordinarily called for. This indicates that the biologist requires the higher homologs in this group. Now the most completely methylated methyl violet is the hexa-methyl compound, which is easily obtained pure and is known to the trade as crystal violet. This dye, therefore, appeared very interesting to the Commission and has been given considerable investigation.

Gentian violet. A poorly defined mixture of violet rosanilins is well-known to biologists under the name gentian violet. The name is not used at present in the dye or textile industries, however, and for this reason the dye is not listed in dye indexes. It apparently applied originally to a certain mixture containing about half dextrin and half dye, the dye being a methyl violet, that is a

mixture of crystal violet with lower homologs of the same series. The statement has been made and often repeated in biological literature that gentian violet is a mixture of crystal and methyl violet; but the looseness of the statement is evident when it is realized that crystal violet is a component of all the deeper shades of methyl violet. It is possible that before the war gentian violet did represent a fairly constant mixture, but there seems to be some doubt even on this point. It is certain that immediately following the war each company used its own judgment as to what to furnish when gentian violet was ordered. As a result there were placed on the market under this name various methyl violets, with or without dextrin, and also crystal violet; of course the purchaser had no knowledge as to what he was obtaining in any given instance.

Under the circumstances the Commission faced a difficult problem in trying to standardize gentian violet. The question was whether to recognize the name at all, or to approve some particular dye or mixture of dyes of this group as gentian violet. The former course was almost impossible because of the wide demand among biologists for a stain labeled gentian violet; it was plain that the stain companies would meet this demand in some way. The second course (unless considerable latitude were recognized) would be entirely arbitrary, inasmuch as no information was available to show which members of this group of dyes were especially needed in histology or bacteriology. Accordingly in the first edition of this book gentian violet was defined as either penta-methyl or hexa-methyl pararosanilin, or else a mixture of methylated pararosanilins composed primarily of the two compounds just named and having a shade at least as deep as that recognized in the trade as methyl violet 2B.

This definition was quite broad and did not exclude anything sold at that time as gentian violet. As a result there are still various different products sold under this name, several of them approved by the Commission. The nature of the imported gentian violet has not been recently investigated; but it is known that there are at least three types of gentian violet on the market, prepared by American concerns. They are as follows: 1) crystal violet, as sold under the labels of the National Aniline and Chemical Co. and of the Hartman-Leddon Co., each of these products being plainly marked "crystal violet" in small letters following the name "gentian violet;" 2) one of the higher methyl violets prepared by Coleman and Bell and labeled "gentian violet improved;" 3) a product manufactured by the National Aniline and Chemical Co. and labeled "gentian violet, Churchman," which is claimed to be a mixture in equal parts of crystal violet and methyl violet 2B. The first two of these types are now being sold under the Commission certification; the latter is not, because it is intended primarily for therapeutic use, and the Commission has never passed upon medicinal dyes.

The situation is not yet regarded as entirely satisfactory. The Commission does not wish to continue to give official recognition to a dye of such indefinite nature as gentian violet; and as soon as users of stains seem to be sufficiently educated in the matter, certification will be refused any dye of this name. Users should specify crystal violet, for bacteriological work and for histological purposes where a deep blue-violet is required; but should order methyl violet 2B in histological procedures where a reddish shade is called for. With this knowledge at hand, gentian violet, as such, is not necessary.

Mallory's modification of the Gram-Weigert method of staining bacteria in tissue:* Use material preferably fixed by the Zenker method, embedded in paraffin and sectioned.

1. Stain sections lightly in alum-hematoxylin.
2. Wash in running water.
3. One percent aqueous solution of eosin Y, 1 to 5 minutes.
4. Wash in water.
5. Anilin methyl violet, one-half to one hour. (There is some question what formula or even what methyl violet has been employed in this method. Undoubtedly one of the bluer methyl violets, such as 2B, is best to employ, or possibly even a crystal violet instead of a methyl violet. The Ehrlich formula, as given on p. 105, is undoubtedly satisfactory.)
6. Wash with water.
7. Lugol's iodine solution, one to two minutes.
8. Wash with water.
9. Blot with filter paper, dehydrate, and clear in several changes of anilin and xylol, equal parts.
10. Wash off with xylol.
11. Mount in xylol-colophonium.

CRYSTAL VIOLET C. I. NO. 681

Synonyms: *Violet C, G,* or *7B. Hexamethyl violet. Methyl violet 10B. Gentian violet.*

(A basic dye: absorption maximum about 591.)
Solubility at 26°C: in water 1.68%; in alcohol 13.87%.

This dye is hexa-methyl-pararosanilin, whose formula is given above as one of the components of methyl violet.

The Commission has made as careful an investigation of this dye as of any other and has become very enthusiastic over it. Methyl or gentian violet is of chief value to the biologist as a nuclear or chromatin stain, having many histological and cytological applications, the one for which it is most commonly used at present being the Flemming triple stain in which it is employed

*From Mallory & Wright (1924) p. 288.

with orange G and safranin—a technic which gives a very high degree of differentiation. (See p. 81.) It is also used for staining amyloid in frozen sections of fresh and fixed tissue, and for staining the platelets in blood while it is much used by the Weigert technic for staining fibrin and neuroglia. The bacteriologist also finds it a useful stain and probably purchases more at the present time than all other biologists together; the chief bacteriological use is in the Gram technic for distinguishing between different kinds of bacteria. A further more recent use is in bacteriological media for inhibiting the growth of Gram-positive organisms, due to its selective bacteriostatic action.

The Flemming and Gram stains have seemed the most delicate procedures for which it is used; so they have been given the most careful study. In the case of the Gram stain it was discovered that there are a score or more different procedures all referred to by the name "Gram" stain, and a study was made of all the methods that were found (see Hucker and Conn 1923). The result of the investigation is to conclude without reservation that crystal violet may be substituted for gentian violet in both the Gram and Flemming technics, and probably for gentian or methyl violet in any of the bacteriological or histological methods for which either stain is designated. If crystal violet can be used in all cases, the advantage is obvious; for it is a definite chemical compound, while methyl and gentian violet are both variable mixtures.

It is of interest to note that in the literature of microscopic technic crystal violet has been specified instead of gentian violet for some special procedures. Worth noting is Benda's crystal-violet-alazirin method for staining chondriosomes, and its modifications by Meves and Duesberg; and also its use in combination with erythrosin by Jackson for staining lightly lignified walls, in which technic it proves more uniform than gentian violet.

Anilin gentian violet (Ehrlich)*

Original statement of formula	*Emended statement*
	Solution A
Sat. alc. sol. gentian violet 5–20 cc.*	Crystal violet (85% dye content) ..... 2.5 g.
Anilin water (2 cc. anilin shaken with 98 cc. water and filtered) 100cc.	Ethyl alcohol (95%) ..... 12 cc.
	Solution B
	Anilin ..... 2 cc.
	Distilled water ..... 98 cc.
	Shake and allow to stand for a few minutes, then filter.
	Mix solutions A and B.

*Altho various anilin water formulae for this dye are known as Ehrlich's, he seems properly to be credited only with the idea of using anilin water in the formula. Various subsequent authors have modified the solution to suit themselves; and as a result the amount of gentian violet recommended in different places varies to the extent shown in the left-hand column above.

The use of crystal violet in this formula is recommended because this dye is more uniform than the various products sold as gentian violet.

Carbol gentian violet (Nicollé)

Formula from Eyre, 2nd ed., p. 92	*Emended statement*
	Solution A
Sat. alc. gentian violet..... 10 cc.	Cyrstal violet (85% dye content)..... 2.0 g.
1% aqu. sol. phenol.....100 cc.	Ethyl alcohol (95%).....10 cc.
	Solution B
	Phenol..... 1 g.
	Distilled water.....100 cc.
	Mix solutions A and B.

The Gram stain for bacteria:

A. Anilin Oil Method

In the original staining technic by Gram the following anilin gentian violet (Ehrlich) was employed

Gentian violet.....	1 part
Alcohol.....	5 parts
Anilin.....	4 parts
Water.....	80 parts

Later procedures call for various modifications of this formula, the most essential one being the shaking up of the anilin with the water separately and filtering before adding to the alcoholic solution of the dye. Gram's original procedure did not call for counter staining; but the various modern modifications all do. An example of such a method is the following:

1. Stain 1 minute in anilin gentian violet made up according to Gram's formula or to the emended statement of Ehrlich's formula as given above (Both formulae should give essentially similar solutions).
2. Blot without washing
3. Immerse for one minute in Lugol's iodine solution:

Iodine.....	1 g.
Potassium iodide.....	2 g.
Water.....	300 cc.

4. Blot without washing.
5. Decolorize in 95% ethyl alcohol for 30 seconds with gentle agitation.
6. Counterstain for 10 seconds with safranin, pyronin, Bismarck brown, or eosin.
7. Wash, and dry.

B. Ammonium Oxalate Method (Hucker)

The stain is as follows:

Solution A

Crystal violet (85% dye content).....	4 g.
Ethyl alcohol.....	20 cc.

Solution B

Ammonium oxalate	8 g.
Water	80 cc.

Mix solutions A and B

The procedure is as above for the anilin oil method except that one must wash briefly in water after the stain and the iodine solution, but must blot dry after washing, particularly just before adding the alcohol. The counterstain recommended is:

Safranin, 90% dye content	0.25 g.
Alcohol, 95%	10 cc.
Water	100 cc.

MacCallum's stain for influenza bacilli in tissues: Material is to be fixed in Zenker's formaldehyde solution, and thin paraffin sections are to be stained as follows:

1. Stain for from 10 to 30 minutes in Goodpasture's stain, as follows:

Ethyl alcohol, 30%	100 cc.
Basic fuchsin (90% dye content)	0.59 g.
Anilin	1.0 cc.
Phenol crystals	1.0 g.

2. Wash in water.
3. Differentiate in 40% formaldehyde, for a few seconds, until the bright red color changes to a clear rose.
4. Wash in water.
5. Counterstain in saturated aqueous picric acid, about 3–5 minutes or less, until section assumes a purplish yellow.
6. Wash in water.
7. Differentiate in 95% ethyl alcohol. This causes the red to reappear; some of it is washed out together with some of the yellow.
8. Wash in water.
9. Stain for about 5 minutes in Stirling's gentian violet. MacCallum does not give the formula of this solution, and its original publication has not been located. The following is suggested as a definite formula probably agreeing with that ordinarily in use under this name:

Crystal violet (85% dye content)	5 g.
Ethyl alcohol, 95%	10 cc.
Grind in mortar, and add too:	
Anilin	2 cc.
Water	88 cc.

10. Wash in water.
11. Immerse one minute in Lugol's iodine solution (see p. 106).
12. Blot dry without washing.
13. Treat in equal parts of anilin and xylol until no more color comes away.
14. Pass thru two changes of xylene.
15. Mount in balsam.

Gram-negative organisms are stained red, Gram-positive blue, and the tissues in shades of red and purple.

Crystal violet with erythrosin in plant anatomy (Jackson): This technic is employed for differentiation of lignified and non-lignified cell walls. The schedule must be varied considerably to suit different lots of material; the general procedure is a follows:

1. Remove paraffin with xylol.
2. Remove xylol with absolute alcohol.
3. Ninety-five per cent alcohol.
4. Crystal violet (about 90% dye content), 1% in distilled water, 15 minutes.
5. Rinse quickly in water.
6. Dehydrate quickly but thoroly in 95% and absolute alcohol.
7. Erythrosin, saturated solution in clove oil, 1 to 5 minutes.
8. Absolute alcohol and xylol, equal parts, 1 to 2 minutes.
9. Xylol.
10. Mount in balsam.

The principal variations in the use of this method are in the length of time for which the stains are allowed to act. The xylem elements in some materials stain satisfactorily in a very few minutes; for other materials a half-hour or more may be necessary. The erythrosin not only stains the non-lignified tissues but tends to replace the violet in the lignified walls; hence its action must be carefully controlled.

Benda's method for staining mitochondria:*

1. Fix for eight days in a modified Flemming fluid (1% chromic acid, 15 cc.; 2% osmic acid, 4 cc.; glacial acetic acid, 3 drops).
2. Wash in water for one hour, then for 24 hours in a mixture of 100 parts pyroligenous acid and one part chromic acid.
3. Transfer to a 2% potassium bichromate solution for 24 hours, run up thru the grades of alcohol to xylol, embed in paraffin and section. Sections should be about 5 μ thick.
4. After removing paraffin, run sections down to distilled water, and place in a 4% iron alum solution for 24 hours.
5. Wash thoroly in water and place for 24 hours in a solution of sulfalizarinate of soda (i. e. alizarin red S); this solution is made by adding one part of saturated aqueous solution of the stain to 80–100 parts of distilled water.
6. Rinse in distilled water and flood with anilin crystal violet (equal parts of anilin water and of a 3% solution of the dye† in 95% alcohol.) Warm until solution steams, keeping it heated for about 3 minutes.
7. Wash in distilled water, transfer to 30% acetic acid for 1–2 minutes, then wash in running water 5–10 minutes.
8. Dry with filter paper, dip a minute in absolute alcohol, clear in bergamot oil, transfer thru xylol and mount as usual.

---

*From Guyer, 1917, p. 144.

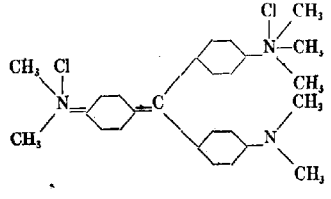
†Probably 90% dye content to be preferred.

METHYL GREEN C. I. NO. 684

Synonyms: *Double green. Light green.*

(*A basic dye; absorption maximum about 633.8.*)

Methyl green is crystal violet into which a seventh methyl group has been introduced by the action of methyl chloride or methyl iodide upon it, forming the compound:*



(*A basic dye.*)

As the seventh methyl group is very loosely attached, there is always some methyl violet present, either because it is not all completely converted into the higher homolog or because it has broken down again. It has been stated that to obtain free methyl green the commercial dye should be shaken in a separatory funnel with amyl alcohol or chloroform, which dissolves the methyl violet. As a matter of fact, however, pure methyl green may not always be desired by the biologist, as the dye owes part of the metachromatic properties for which it is prized to the presence of small amounts of the violet compound.

Methyl green is at present one of the most valuable nuclear stains known to the histologist, and is widely used as a chromatin stain by the cytologist. On the other hand it has been used by Galeotti as a cytoplasm stain following acid fuchsin and picric acid. In the Ehrlich-Biondi technic it is used to stain nuclei in contrast to acid fuchsin; while Bensley employs it to stain chromatin in contrast to acid fuchsin which stains the mitochondria. (See p. 99.) It is an ingredient of the Ehrlich triacid mixture (with orange G and acid fuchsin) for staining blood smears. Botanists find it a valuable stain, combined with acid fuchsin, for lignified xylem. One of its most valuable uses today is in the Pappenheim stain, (see p. 119) in which it is combined with pyronin and used for staining the gonococcus and mast cells as well as by Unna in studying chromolysis. It is also a useful chromatin stain for protozoa, and is employed in weak acetic acid solution for staining fresh material beneath the coverglass.

*This ordinarily occurs in trade as a zinc double salt.

When the foreign supply of dyes was first shut off, this stain proved one of the most difficult to obtain in satisfactory quality, largely due to the looseness with which the seventh methyl group is attached and the resulting instability of the compound. At first certain green dyes of an entirely different nature were furnished, but as soon as an investigation of the dye was begun manufacturers proved perfectly able to produce methyl green; the difficulty came in obtaining the right degree of purity. Samples were finally furnished so pure that they lacked completely the necessary metachromatic staining quality; and it proved necessary to add a certain small percentage of the violet dye to obtain the proper results. This problem seems to have been solved at present and satisfactory methyl green is available. The chief problem now is to standardize it. With other stains this can ordinarily be done on the batch basis, approving some batch large enough to meet the demand for a period of years. With methyl green this cannot safely be done, on account of its instability. Hence large batches are impractical; and the stain ought to be sold with the caution that the dye does not keep indefinitely without change.

ACID VIOLET

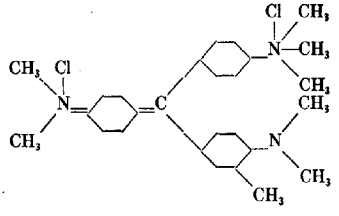
There are various dyes on the market known as acid violet with some shade designation. These dyes vary extremely in composition and they are listed under various Colour Index numbers. They are in general sulfonated violet dyes of the rosanilin group, some of them simple methylated pararosanilins, others benzylated compounds.

Some acid violet—its exact identity uncertain—has been employed by Bailey in cytological studies on the human pituitary gland; also by Maurer and Lewis for staining similar tissue from the pig. These authors employ it alone, as a counterstain to basic fuchsin, mixed with acid fuchsin, and in neutral stain combination with safranin. It is very unfortunate that any biologist should have been furnished a stain labeled merely acid violet; the term is too indefinite for identification.

IODINE GREEN C. I. NO. 686

Synonym: *Hofmann's green.*

This dye is closely related to methyl green, the generally accepted formula being:



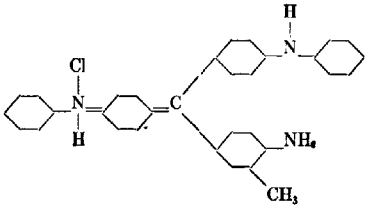
(*A basic dye.*)

Iodine green is a nuclear or chromatin stain which has selective properties that make it of value in certain special procedures. It is used by Giaccio for nervous tissue in combination with acid fuchsin and picric acid; and by Lefas as a blood stain in combination with acid fuchsin. It is used by Zimmermann with basic fuchsin for staining chromatin in plant tissue; while together with acid fuchsin it is occasionally used by botanists for staining lignified xylem. It is also used for staining mucin and amyloid, having the property of giving the latter a red instead of a green color.

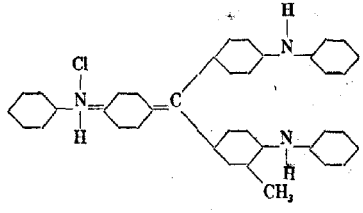
SPIRIT BLUE C. I. NO. 689

Synonyms: *Gentian blue. Anilin blue, alcohol soluble. Night blue. Lyons blue. Paris blue.*

This is a mixture of di-phenyl rosanilin



and tri-phenyl rosanilin:



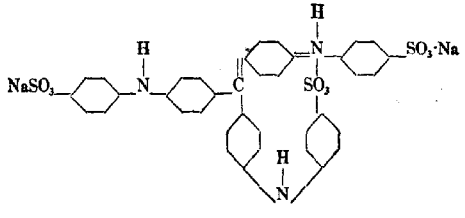
(Basic dyes; absorption maximum of spirit blue 2R about 581 in alcohol.) *

Solubility at 26°C: in water nil; in alcohol 1.10%

It is used in strong alcoholic solution, in contrast to carmin, in staining embryonic tissues; it brings out growing nerve fibers well.

METHYL BLUE C. I. NO. 706

Synonyms: *Cotton blue. Helvetia blue.*



(An acid dye; absorption maximum about 607.)

On account of the sulfonic groups, this dye is strongly acidic and makes a good counter stain. It is used by Mann with eosin for staining nerve cells; and by Dubreuil, combined with picric acid, in contrast to a red nuclear stain such as carmin or safranin. It can apparently be substituted frequently for the following type, having been thus tried in the Mallory connective tissue stain.

Mann's methyl-blue-eosin:*

1% methyl blue† solution (aqueous?)	35 cc.
1% aqueous eosin† solution	35 cc.
Distilled water	100 cc.

Procedure:

1. Material is fixed in Mann's solution (1 g. picric acid, 2 g. tannin in 100 cc. saturated $HgCl_2$, NaCl solution).

*From Krause, (1926–7), p. 849.

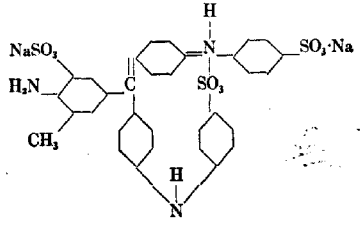
†Information as to exact dye content of samples best to employ is not now available.

2. Sections are stained 24 hours in the above staining solution.
3. Wash in water.
4. Dehydrate in alcohol.
5. Place in following solution: 1% NaOH (in absolute alcohol) 4 drops, absolute alcohol 50 cc. The sections become red in this solution.
6. Wash quickly in absolute alcohol.
7. Bring into water and remove excess blue stain; allow to stand two minutes.
8. Place in water slightly acidified with acetic acid, in which they become blue again and no more stain comes out.
9. Dehydrate and mount as usual.

ANILIN BLUE, W. S. (i.e., water soluble) C. I. NO. 707

Synonyms: *China blue. Soluble blue 3M* or *2R. Marine blue. Cotton blue. Water blue.*

This is a mixture of the tri-sulfonates* of tri-phenyl para-rosanilin (C. I. 706) and of di-phenyl rosanilin. The latter is:



(An acid dye; absorption maximum of water blue 2B about 546.5.)

Anilin blue W. S. should be regarded as a group of dyes rather than as a simple dye. The composition of the various commercial products sold under this name is uncertain, and no method seems to be known for controling the process of manufacture so as to yield a constant product.

Some dye or dyes of this group are widely used in histology, having valuable counterstaining properties. It is also of use as an indicator, due to the disappearance of the color upon complete neutralization, as in the case of acid fuchsin. As an indicator, however, it has the disadvantage that the blue color is but slowly restored upon addition of acid.

Its chief histological uses are: by Stroebe and Huber as a cytoplasm stain preceding safranin; by Galli for axis cylinders; fre-

*The location of the sulfonic groups is uncertain.

quently by botanists as a contrast for safranin in vascular plant tissue, or for magdala red in algae; and very widely by pathologists in the Mallory connective tissue stain, in which it is combined with orange G and acid fuchsin; by Unna in contrast to orcein for staining epithelial sections, and in studying the process of chromolysis.

Under the name of Poirier's blue or cotton blue C$_4$B, Scales (1922) has employed a dye of this type as a bacterial stain, obtaining results similar to the Gram stain. Unfortunately the dye is no longer obtainable from the source from which he obtained it, and thus far the nature of the dye he employed has not been determined.

On account of the lack of constancy in this group, any one publishing a technic calling for one of these dyes should be very careful to give all the information obtainable from the label and should specify the source from which the sample used was obtained. If a Commission certified stain is employed, its certification number should of course be given.

Mallory's connective tissue stain:

1. Fix in Zenker's fluid.
2. Embed in celloidin or paraffin.
3. Stain sections in a 0.5% aqueous solution of acid fuchsin* for five minutes or longer, depending on the freshness of the tissue.
4. Transfer directly to the following solution and stain from ten to twenty minutes or longer:

Aniline blue W. S. (preferably certified product)	0.5 g.
Orange G (80-85% dye content)	2.0 g.
1% Aqu. Sol. of phosphomolybdic acid	100.00 cc.

5. Wash and dehydrate in several changes of 95%, then absolute alcohol.
6. Clear in xylol.
7. Xylol colophonium or balsam.

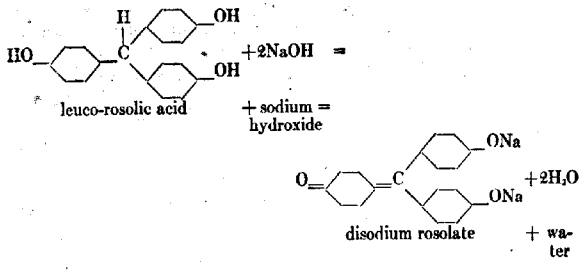
Collagen fibrils, reticulum, amyloid, mucus, and certain other hyaline substances stain blue; nuclei, cytoplasm, fibroglia fibrils axis cylinders, neuroglia-fibers, and fibrin red; red blood-corpuscles and myelin sheaths yellow; elastic fibers pale pink or yellow.

c. Hydroxy Phenyl Methanes (Rosolic Acids)

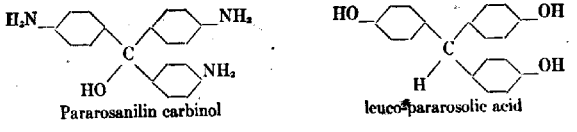
The rosolic acid dyes, as stated above, are tri-phenyl methane derivatives in which the amino groups of the rosanilins are replaced with hydroxyl groups, thus giving them acidic instead of basic character. The compounds of this group are not very important as dyes and are scarcely used as stains. The greatest interest of the biologist in them is due to their use as indicators, since in acid solution the quinoid ring disappears and the com-

*This step may be preceded to good advantage by light staining in Delafield's hematoxylin or Mayer's hemalum; this causes the nuclei and smooth muscles to stain a brownish color.

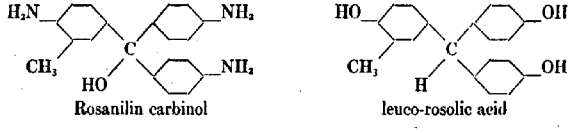
pound becomes colorless, while alkali changes it back to the colored form. Thus:



There is considerable confusion in the nomenclature of these dyes, as the names employed may be used in a strict chemical sense or in a looser sense in practice. Chemically there are two rosolic acids, which are related just as are rosanilin and pararosanilin. Pararosolic acid differs from pararosanilin, only in having hydroxyl groups in place of the amino groups:



Rosolic acid, on the other hand, is a mono-methyl derivative, and bears the same relation to rosanilin:



Now the dye to which the name rosolic acid or aurin is generally given in practice is a mixture consisting of both rosolic acid and pararosolic acid together with other closely related compounds. This dye is:

AURIN OR ROSOLIC ACID C. I. NO. 724

A mixture of rosolic acid and pararosolic acid, with oxidized and methylated derivatives of the latter. This product is of considerable use as an indicator.

This dye is employed by Pappenheim in a decolorizing solution following carbol fuchsin. The method is intended to safeguard

against confusion of the smegma and other acid-fast bacteria with the true tubercle bacillus. After the usual carbol fuchsin stain, the decolorizing is done with 1% rosolic acid in absolute alcohol, saturated with methylene blue and containing 20% glycerol.

Corallin yellow is the name given to the sodium salt of this dye.

No other dyes of this group have biological use. Two others perhaps deserve mention:

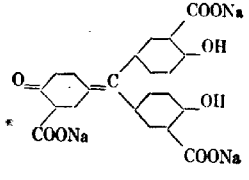
CORALLIN RED C. I. NO. 726

Synonym: *Aurin R.*

Apparently a compound dye, the pararosanilin salt of pararosolic acid.

CHROM VIOLET C. I. NO. 727

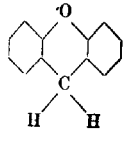
A carboxyl derivative of pararosolic acid:



CHAPTER VII

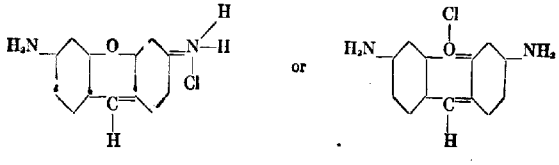
THE XANTHENE DYES

THE group of compounds known as xanthene dyes comprises a number of basic and acid dyes and quite a series of indicators. In fact, the most valuable indicators known to the chemist fall in this group. They are derivatives of the compound xanthene:

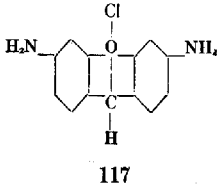


1. THE PYRONINS

The pyronins are methylated di-amino derivatives of xanthene. They are closely related to the diphenyl methanes and are sometimes classed with them, as they have a carbon atom attached to two benzene rings, and show the same tendency toward quinone structure. Their formula, on the other hand is like that of the oxazins except that the nitrogen of the central ring is replaced by a methenyl (CH) radical. Like the oxazins, the atomic grouping may be assumed to be in either the paraquinoid or the orthoquinoid form, thus:

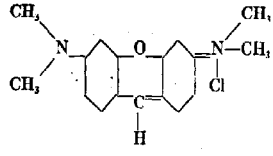


Another arrangement of the atoms is possible in which no quinoid ring exists, namely:



This latter form might also be assumed for the oxazins and thiazins as well, and this type or formula is frequently used for the azins; but the xanthene dyes are more often represented in this form. If this formula is adopted the quinoid ring cannot be accepted as their chromophore. For this reason one of the quinoid formulae seems preferable; and for the sake of uniformity the paraquinoid form will be given in the following pages. It must be remembered, however, that the other formulae are equally admissible; and it is possible that the compounds occur in two or even all three of the different forms.

PYRONIN G C. I. NO. 739

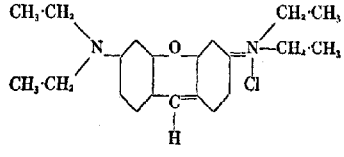


(*A basic dye; absorption maximum about 545.*)

Solubility at 26°C: in water 8.96%; in alcohol 0.60%.

This dye, finding occasional application as a biological stain in pre-war days, is now difficult to obtain. It is not manufactured in America. Fortunately the following may be substituted for it.

PYRONIN B C. I. NO. 741



(*A basic dye; absorption maximum about 550.*)

Solubility at 26°C: in water 0.07%; in alcohol 1.08%

This dye differs from pyronin G only in that it is an ethyl instead of a methyl derivative. As a result it is very slightly deeper in shade but has almost identically the same staining behavior. Investigations recently carried on by the Commission indicate that it can replace pyronin G in the Pappenheim stain and probably in all its other uses. This is very encouraging, for it is much more easily prepared and a very satisfactory product of American manufacture is now available.

This dye finds its principal use as a stain in the Pappenheim combination, where it is employed with methyl green for staining basophile elements, especially the mast cells, and for staining the gonococcus in smears of pus. It is also used sometimes as a counterstain in the Gram technic for bacteria (see p. 106); and by Ehrlich as a component of certain "neutral" stains.*

The Pappenheim-Saathof stain. The formula for this combination as generally given in the literature calls for 0.15g. methyl green to 0.5g. of pyronin. American pyronins, however, are so much more concentrated than those available before the war that a different formula is necessary. The following is now recommended:

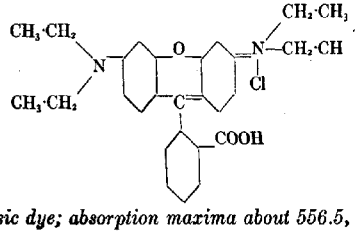
Methyl green (dye content 55-60%)	1.0 g.
Pyronin B (certified product)	0.25 g.
Ethyl alcohol, 95%	5. cc.
Glycerol	20. cc.
Phenol, 2% aqueous sol	100. cc.

2. THE RHODAMINES

The rhodamines are similar to the pyronins except that there is a third benzene ring attached to the central carbon atom and attached to this ring is a carboxyl group in the ortho position. This latter group, altho of acid tendency, does not counteract the basic action of the amino groups, so the dyes are basic in character. Only one of them is of any significance to the biologist, namely:

RHODAMINE B C. I. NO. 749

Synonyms: *Rhodamine O. Brilliant pink.*



(*A basic dye; absorption maxima about 556.5,* [*519*])

Solubility at 26°C: in water 0.78%; in alcohol 1.47%

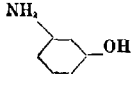
A rhodamine, probably the above dye, has been used by Griesbach with osmic acid to fix and stain blood simultaneously; by Ehrlich as a component of "neutral" stain mixtures; by Rosen

*For literature references to the procedures mentioned in this chapter see pp. 174 to 195 and 204 to 216.

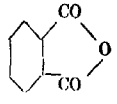
for histological work in contrast to methylene blue; and by others in contrast to methyl green.

A somewhat different dye, known as Rhodamine S (C. I. No. 748) has been mentioned in the same connection and may have been used for some of the above mentioned purposes. It is not a true rhodamine, however, but belongs to a closely related group of compounds, the succineins; for it does not have the three benzene rings, the radical $C_6H_4 \cdot COOH$ being replaced by $C_2H_4 \cdot COOH$.

In practice the rhodamines are prepared not from xanthene but by the condensation of two molecules of meta-amino phenol,



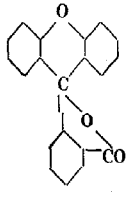
with one of phthalic anhydride:



This shows their close relation to the next group of dyes, namely the fluorane derivatives, which as will be seen are also prepared from phthalic anhydride. In fact these two groups of dyes, acid and basic respectively, are related in exactly the same way as the rosolic acids and the rosanilins, the one group having hydroxyl radicals where the other has amino groups.

3. FLUORANE DERIVATIVES

Fluorane is not a dye, but is a very important compound in dye chemistry. It is a derivative of phthalic anhydride, and contains a xanthene ring (five C atoms and one O atom) as well as a lactone ring (four C atoms and one O atom) besides three benzene rings; thus:



The fluorane dyes are derivatives of this by the introduction of hydroxyl groups into two of the benzene rings at the para position

to the central carbon atom and the further introduction of halogen atoms at various positions in all three benzene rings.

It proves convenient here to class these compounds with the xanthene dyes. They may, however, be equally well considered tri-phenyl-methane dyes, as can be seen by a glance at the formula of any of them; in fact they are generally so considered by the chemists. To the biologist they stand in a distinctly different class from the tri-phenyl-methanes; and for that reason are treated here instead of in the preceding chapter. The dyes of this group are also, and equally correctly, spoken of as the fluorescein derivatives, fluorescein being, as will be seen on p. 123, a salt of di-hydroxy-fluorane. Sometimes, moreover, the fluorescein dyes are referred to as the eosins. This term to cover the whole group is hardly justified, as it is better to call eosins only the dyes definitely so named below.

The fluorescein dyes are all similar in their action, but with certain decided differences. In order for the reader to obtain a real understanding of these stains and their behavior, a general discussion of the whole group is necessary.

The dyes in this group most important to the biologist are: eosin Y, C. I. No. 768 (also called eosin yellowish or eosin water soluble); ethyl eosin, C. I. No. 770 (also called eosin alcohol soluble); eosin B, C. I. No. 771 (also called eosin bluish); erythrosin B, C. I. No. 773; phloxine, C. I. No. 774; rose bengal, C. I. No. 779. It is not yet absolutely certain which of these dyes is most suitable for any particular purpose; but recent investigation has given considerable information on the subject. The difference in behavior of the different dyes of this group seems to depend upon two factors: difference in color and difference in acidity. The relation between these two factors and chemical structure has recently been discussed by Conn and Holmes (1926); by reference to which paper it will be seen that in color the dyes above listed increase in depth in the following order: eosin Y, ethyl eosin, eosin B, erythrosin B, phloxine, rose bengal. In the case of each of these, the color is lighter or deeper according to the number of halogen atoms present. Eosin B is the strongest acid in the group, eosin Y the next strongest; erythrosin is weaker, phloxine weaker than eosin and possibly weaker than erythrosin; while rose bengal is the weakest of all.

To interpret this information into terms of actual use, it is necessary to remember that there are two main types of histological procedures in which eosin is used: first as a general counterstain (usually in alcoholic solution) following a basic dye; secondly, as a cytoplasm stain (always in aqueous solution) preceding a basic dye. In the first of these types of procedure it is important that the dye be one with diffuse staining properties and with a color showing good contrast to the basic dye employed (generally methylene

blue or hematoxylin). The more acid and lighter colored dyes in the series (eosin Y, ethyl eosin, and eosin B) seem to possess these properties to the greatest extent and accordingly to be best adapted to such procedures. The particular dye to choose depends undoubtedly on the exact shade desired.

The second type of procedure (i. e. preceding a basic dye) is represented by the Mallory phloxine-methylene-blue stain and the Held erythrosin-methylene-blue technic. In such procedures as these, both dyes are used in aqueous solution; and there is therefore much more opportunity for chemical interaction between the acid dye already in the tissue and the basic dye subsequently added than when the acid dye is used in alcoholic solution. (It is well known that acid and basic dyes in alcoholic solution do not form insoluble reaction products.) In procedures like the Mallory technic, there seems to be a tendency (possibly on account of this interaction) for the acid dye to stain the cytoplasm too weakly when followed by a basic dye, in case the very acid eosin Y is used. Thus Held employs erythrosin, and Mallory who formerly specified eosin has recently discovered that his original eosin was not a true eosin Y, phloxine giving better results in his technic than one of the true eosins. Now phloxine and erythrosin are not only deeper in color than eosin Y, but are also less strongly acidic; and it is possible that their chemical nature rather than their color may be the decisive factor in determining their superiority for such procedures as this. Rose bengal is even deeper in color, and is still less strongly acidic; in fact, it seems to be too purple to contrast well with methylene blue and of such a weakly acid character that it tends to remain in the nuclei when used by the technic in question. It might prove valuable preceding some basic dye; but it has been found to give poor results preceding methylene blue in the Mallory technic.

In the procedure for staining bacteria in soil, as developed by Conn (1918) and by Winogradsky (1924), the matter is still further complicated by the fact that the dye must be of such a color and such a strong acid as to stain the bacteria, but not the dead organic matter present.

Another factor of much importance in the staining action of these dyes is the amount of mineral salt present. It has been recently shown by Conn and Holmes (1928) that the intensity with which a dye of this group can stain bacteria may be greatly increased by adding a minute amount (0.001 to 0.1%) of some mineral salt such as $CaCl_2$. This matter is discussed in Chapter II.

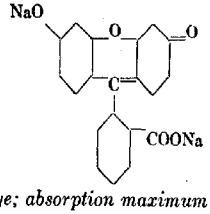
There has been in the past much mislabeling of the dyes of this group. Thus it is evident that the eosin used by Mallory when he first worked out his eosin-methylene-blue technic must have been a phloxine or some closely related dye rather than a true eosin. There is evidence that erythrosin B and phloxine have been put on

the market labeled eosin bluish; so has a blend of eosin Y shaded with some one of the higher members of the group. Phloxine has been sold as magdala red and is even now sometimes marketed under that name both by American and foreign concerns. All this has caused much confusion; and dealers in stains should use greater caution in the matter in the future.

FLUORESCEIN C. I. NO. 766

Synonym: *Uranin.*

This is the simplest of the fluorane dyes, and is the mother substance of the eosins. The composition of its sodium salt is:



(*An acid dye; absorption maximum about 490.*)

Solubility at 26°C: in water 50.2%; in alcohol 7.19%.

Fluorescein is a yellow dye of very low tinctorial power, and hence of no value as a stain. It is on the other hand extremely fluorescent, the greenish yellow fluorescence being detectable in extremely high dilution. On account of this latter property the dye is used to determine the possibility of contamination from some suspected source getting into a neighboring water supply.

EOSIN, Y (i.e., YELLOWISH) C. I. NO. 768

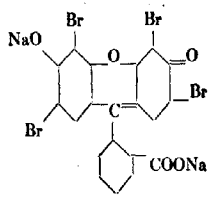
Synonyms: *Water soluble eosin. Eosin W* or *WS.*

Various shades denoted: Eosin G, Y extra, S extra, J extra, B extra, GGF, 3J, 4J, KS, DH and JJF.

(*An acid dye; absorption maximum about 516.*)

Solubility at 26°C: in water 44.20%; in alcohol 2.18%

This dye is typically tetrabrom fluorescein:



but the mono- and di-brom derivatives are also known and frequently occur in eosin. This affects the shade, as the more bromine present the redder the dye. It is plain that various mixtures of these compounds are on the market; but it has not yet been determined which are more suitable for biological purposes. Considerably more work on eosin is needed than has been done at the present time. From the name "water soluble eosin" it is often assumed that this dye is not soluble in alcohol. This is not true, however.

Yellowish eosin is one of the most valuable plasma stains known. It is used in various technics for staining the oxyphile granules of cells (i.e., the granules having special affinity for acid dyes); these cell elements, in fact, being often called eosinophile granules because their presence was first recognized thru the use of this dye. It is often employed as a counterstain for hematoxylin and the green or blue basic dyes; as for example by List with methyl green. It is used by Mann mixed with methyl blue as a tissue stain (see p. 112); and by Teichmüller for staining sputum before staining with methylene blue. At the present time one of the uses for which it is in greatest demand is as a blood stain in the technic of Romanovsky, with its various modifications, in which it is combined with methylene blue to form a "neutral" stain.

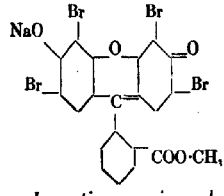
Eosin as counterstain, following a basic dye, such as hematoxylin:

Eosin Y (about 85% dye content)	0.1–0.5 g.
Ethyl alcohol, 95%	25 cc.
Water	75 cc.

METHYL EOSIN C. I. NO. 769

Synonym: *Eosin, alcohol soluble.*

This is the methyl ester of yellowish eosin, the sodium salt of which is:



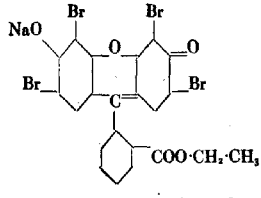
(*An acid dye; absorption maxima about 520*, [*485.5*])

There is no evidence that this dye has been furnished to biologists as a stain. The alcohol soluble eosin known in the biological laboratory is apparently the following.

ETHYL EOSIN C. I. NO. 770

Synonyms: *Eosin, alcohol soluble. Eosin S.*

This is similar to methyl eosin, but is the ethyl ester:



(*An acid dye; absorption maxima about 523.5*, [*487*])

Solubility at 26°C: in water 0.03%; in alcohol 1.13%

Ethyl eosin is apparently the alcohol soluble eosin sold by biological supply houses before the war. The imported product seems to have been constant, and is still the same dye. Following the war it was difficult to secure this dye in America, and one stain company for a few years furnished the color acid of eosin Y when alcohol soluble eosin was ordered. (The color acids of all these dyes are alcohol soluble but almost insoluble in either hot or cold water; methyl and ethyl eosin, on the other hand, are slightly soluble in cold water but strongly soluble in hot water and alcohol.) This error has now been corrected, and true ethyl eosin as obtainable from one or two American manufacturers, is on the Commission certification basis, and can be ordered from any biological supply house. It should be ordered as ethyl eosin rather than alcohol soluble eosin, as the latter name is less definite.

Ethyl eosin is a valuable counterstain after Delafield's hematoxylin. Preceding methylene blue, it is employed in demonstrating Negri bodies in the central nervous system of rabid animals.

Harris' technic for demonstrating Negri bodies in smears:

1. Make smears of the nervous tissue to be examined between two microscopic slides.
2. Fix in methyl alcohol one minute.
3. Wash briefly in water.
4. Immerse in old (i. e. 2 months or more) saturated solution of ethyl eosin in 95% alcohol, from 1–3 minutes.
5. Wash 2–3 seconds in water.
6. Immerse in fresh solution of Unna's alkaline methylene blue 5 to 15 seconds.
7. Wash briefly in water.
8. Decolorize in 95% alcohol; follow with absolute alcohol.
9. Pass thru xylol and mount in balsam; or blot and dry in air.

Technic for demonstrating Negri bodies in sections: This technic has been worked out by W. D. Stovall of Madison Wis., but has not been published. The details were furnished in a personal letter. It seems to give much more satisfactory preparations than the smear method, as above. By this method the major portion of the tissue takes a pink hue with a slightly bluish background. The nucleoli stain pale blue, the Negri bodies a terra cotta to cardinal red.

A. Embedding and sectioning

1. Cut specimens from the hippocampus major into pieces 3–5 m.m. in thickness.
2. Place these pieces between squares of ordinary writing paper, cut end next to the paper, and immerse in acetone for 2–4 hours.
3. Remove the paper and place the fixed tissue in soft paraffin for one hour.
4. Replace the soft paraffin with hard paraffin and allow to stand one hour.
5. Mount on microtome block and section, cutting specimens 5 μ thick.
6. Float onto glass slides and fix by heat over a Bunsen burner. This heating must be done gently and just to the melting point of the paraffin. This should be allowed to solidify and again gently heated. Repeat this two or three times.

B. Staining

1. Wash in xylol.
2. Remove xylol with absolute alcohol.
3. Pass into 95% alcohol, and then to 70%.
4. Remove to distilled water, washing in each fluid until all of the previous fluid is removed.
5. Stain one minute in a saturated solution of ethyl eosin in *neutralised* 95% alcohol. Different batches of eosin vary greatly in their ability to stain Negri bodies. Some eosins fail to stain them altogether. In the latter case,

one can stain the bodies successfully by adding to the saturated alcoholic solution 1% acetic acid. The amount of acid to be added varies with each batch of stain and will have to be determined for the stain.

6. Wash in water.
7. Stain one-half minute in Loeffler's methylene blue.
8. Wash in water.
9. Differentiate in water acidulated with acetic acid (12 drops of glacial acetic acid to one ounce of water).

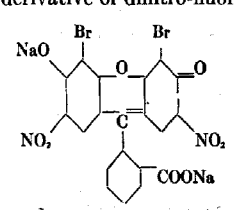
This is the crucial point of the staining. The acid serves to fix the eosin in the Negri bodies and to remove the excess blue from the pyramidal cells. One will have to become familiar with the proper staining methods to know how to use this differentiating fluid.

10. Run back thru the alcohols into xylol to clear.
11. Mount in balsam.

EOSIN, BLUISH C. I. NO. 771

Synonyms: *Eosin BN, B, BW,* or *DKV. Safrosin, Eosin scarlet B* or *BB. Scarlet J, JJ, V. Napolin G. Caesar red.*

This is a dibrom derivative of dinitro-fluorescein.

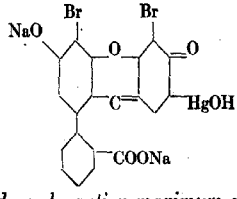


(An acid dye; absorption maxima about 521.5, [486])
Solubility at 26°C: in water 39.11%: in alcohol 1.87%

It has occasionally been called for as a counterstain in some histological procedure (as, for example, in aqueous solution following Mayer's hemalum), but is not in general very valuable. Ordinarily, if a shade deeper than eosin Y is desired, better results can be obtained with erythrosin, phloxine or rose bengal than with eosin B. Coleman and Bell, in fact, puts on the market at present a product labelled "eosin, bluish blend," which is a mixture of eosin Y with some one of the dyes just named; it is very satisfactory for certain staining procedures, but must not be confused with true eosin B.

MERCUROCHROME 220.

This is a proprietary name applied to a fluorescein derivative closely related to eosin: dibrom-oxy-mercuri-fluorescein.



(*An acid dye; absorption maximum about 524.*)

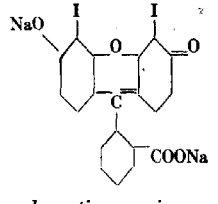
Solubility at 26°C: in water 11.10%; in alcohol 1.87%

The dye has been widely advertised as a disinfectant especially for the skin; and there has been considerable discussion in the literature as to its actual value for the purpose. It has staining properties not unlike an erythrosin, or phloxine. Baldwin (1928) states that it may be used in 2% aqueous solution in place of eosin, especially for blood work and for tissues after Zenker fixation; thus employed it is more intense than eosin and has a stronger affinity for cytoplasmic structures. Detwiler and McKennon (1929) employ it in concentration of from 1:500,000 to 1:1,000,000 as a fungicidal agent for the treatment of amphibian embryos.

ERYTHROSIN, YELLOWISH C. I. NO. 772

Synonyms: *Erythrosin R* or *G. Pyrosin J. Dianthine G. Iodo-eosin G.*

This is a fluorescein in which there are two substituent iodine atoms instead of four bromine atoms as in yellowish eosin.

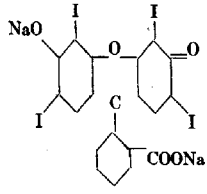


(*An acid dye; absorption maximum about 510.5.*)

ERYTHROSIN, BLUISH C. I. NO. 773

Synonyms: *Erythrosin B. Pyrosin B. Eosin J. Iodo-eosin B. Dianthin B.*

This is the tetraiodo compound corresponding to the tetrabrom compound of typical eosin.



Erythrosin has some use as an indicator. It is also employed as a contrast stain for hematoxylin and certain blue and violet nuclear stains. Held uses it, preceding methylene blue, as a plasma stain for nerve cells (see p. 69). The technic of Jackson (see p. 108) calls for it as a counterstain to crystal violet in plant histology. It is employed by Winogradsky for staining bacteria in soil. For these purposes probably the tetra-iodo compound (i. e., erythrosin bluish) is desired; but the literature is vague on the subject.

A sample of erythrosin of pre-war origin that was labeled magdala red has been examined by the Commission. This mislabeling undoubtedly explains Chamberlain's results already mentioned (page 84) in staining algae. Chamberlain, it will be recalled, was able to obtain good results with a low priced product called magdala red but not with the high priced stain called magdala red *echt*.

Winogradsky's stain for bacteria in soil:

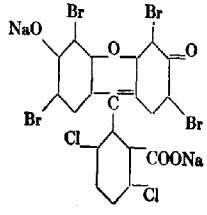
Erythrosin (B?).....1 g.
Phenol, 5% aqu. sol.....100 cc.

This solution is employed for staining films prepared from the various fractions obtained by centrifugalizing soil infusion.

If in the manufacture of fluorescein, dichlor or tetrachlor phthalic acid is used for condensation with resorcin, a series of halogenated derivatives may be obtained differing from those just named by having chlorine atoms in the phthalic acid residue of the molecule as well as in the resorcin residue. These compounds are slightly deeper in shade than the corresponding derivatives of simple phthalic acid; and they are generally regarded as being of more pleasing shade than the ordinary fluorescein dyes. The phloxines and rose bengals are the best known of these dyes.

PHLOXINE C. I. NO. 774

Synonyms: *Erythrosin BB*, or *B extra*. *New Pink*.



(*An acid dye; absorption maximum about 535.7*, [*497.1*])

Solubility at 26°C: in water 50.9%; in alcohol 9.02%.

This dye and the following are often denoted interchangeably phloxine or phloxine B, and there is some question as to whether biologists have always been furnished the same type. The phloxines of American manufacture at present on the market are the following type.

PHLOXINE B C. I. NO. 778

Synonyms: *Phloxine TA*, *N*, or *BB*. *Cyanosine*. *Eosin 10B*.

(*An acid dye; absorption maximum about 539.5*, [*500.8*])

This dye differs from C. I. No. 774 in having four instead of two chlorine atoms in the phthalic acid residue of the molecule. It is the type of phloxine recently submitted to the Commission for certification by two different stain companies; and altho some of the procedures given below may have been worked out originally with the other type of phloxine, this type has been found to give satisfactory results.

Unna uses phloxine in combination with several other acid dyes in studying the process of chromolysis. The dye has seldom been specified for biological work; yet there is reason to believe that it is a more valuable stain than anyone has realized in the past, and that it has frequently been used under other names.

Chamberlain (1924 page 58) mentions having used it successfully in place of magdala red in staining algae. His original technic called for magdala red; but true magdala red does not serve his purposes. Inasmuch as erythrosin (see above) was evidently sold in the past as magdala red and Chamberlain can duplicate his original results with phloxine, the chances are that some of the magdala red formerly available was either phloxine or else that

phloxine and erythrosin give similar results by Dr. Chamberlain's technic.

After the war Dr. F. B. Mallory like many others found it difficult to obtain an eosin of either domestic or foreign origin which would give consistently reliable results by his eosin-methylene blue method. After testing out, on the advice of a member of the Commission, a series of eosins and closely related dyes he wrote that phloxine is "the best *eosin* I have yet found for use in the eosin-methylene blue stain for paraffin sections of tissues fixed in Zenker's fluid."* Here again is a case where phloxine apparently was obtained before the war under an incorrect name and the incorrect name used in the publication of a well-known technic.

Chamberlain's method of staining algae:

1. Fix material in formalin acetic acid, or in chromo acetic acid.
2. Wash thoroly in water and place in 10% glycerin.
3. Wash thoroly in 95% ethyl alcohol until all traces of glycerin are removed.
4. Stain over night in a 1% solution of phloxine (dye content about 80%) in 90% ethyl alcohol.
5. Pour off stain and wash for a minute in 90 or 95% alcohol.
6. Stain 1 minute in a 1% solution of anilin blue W. S. (C. I. No. 707) in 90% alcohol.
7. Treat briefly with weakly acidulated alcohol. This alcohol is to be prepared by keeping it in a vessel into which hydrochloric acid had been placed and then removed as completely as possible by draining. Remove this acidulated alcohol 3–4 seconds after pouring over material.
8. Wash with two or three changes of 95% alcohol.
9. Place in absolute alcohol 4 or 5 minutes.
10. Examine under microscope. If chromatophore is a clear clean blue, the pyrenoids and nucleoli a bright red, the stain is good. If the red has been drawn out too much by the acid, put the material into phloxine 30 minutes or so, wash in 95% alcohol and dehydrate again. If the red is bright but the blue too weak, repeat the staining in anilin blue and dehydrate once more.
11. Place in 10% Venetian turpentine in an exsiccator until the turpentine is of the consistency of pure glycerin. (2 days or so).
12. Mount in a few drops of Venetian turpentine.

Mallory's phloxine and methylene-blue stain:

Fix in Zenker's fluid.

1. Stain paraffin sections in a 5% aqueous solution of phloxine (dye content about 80%) for twenty minutes or longer. Sometimes it is advisable to get a deeper phloxine stain by placing the sections in the paraffin oven for thirty to sixty minutes.
2. Wash in water to get rid of excess phloxine.
3. Stain for 30 minutes in the following borax methylene blue solution diluted in 10 parts of water:

Methylene-blue, Med. (90% dye content)	1 g.
Borax	1 g.
Water	100 cc.

*Quoted from personal letter.

Pour the solution off and on the sections several times.

4. Wash in water.

5. Differentiate and dehydrate in a dish of 95% alcohol, keeping the section in constant motion, so that the decolorization shall be uniform. Control the result under the microscope. When the pink color has returned to the section and the nuclei are still a deep blue, finish the dehydration quickly with absolute alcohol.

6. Absolute alcohol.

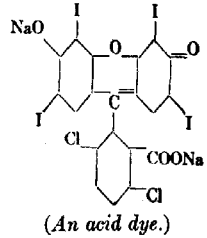
7. Xylol.

8. Xylol colophonium, or balsam.

For celloidin sections use absolute alcohol, blot, and pour on xylol, or beechwood creosote followed by xylol; repeat the last two steps until the specimen is clear.

ROSE BENGAL C. I. NO. 777

Various shades denoted: Rose bengal *N*, *AT*, *NT*, *NTO*, *B* and *6*.
Synonym: *Rose SA*.

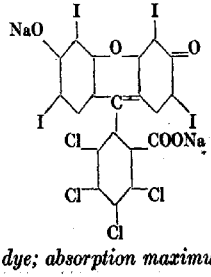


(*An acid dye.*)

This particular rose bengal apparently has not been employed by biologists. The dye of that name sold by stain companies at present is the following.

ROSE BENGAL C. I. NO. 779

Various shades denoted: Rose bengal extra, 3B Conc., N extra, *B* and *2B*.



(*An acid dye; absorption maximum about 548*).
Solubility at 26°C: in water 36.25%; in alcohol 7.53%

This dye has a pleasing deep pink color; and altho an acid dye it proves to have considerable affinity for bacterial protoplasm, and to have good selective properties when used as a bacterial stain. It has been recommended (Conn 1917) for staining bacteria in soil suspensions. It has also been used as a cytoplasm stain following hematoxylin.

Conn's technic for staining bacteria in soil:

1. Make a suspension of soil in nine times its weight of a 0.015% solution of gelatin.

2. Put a drop of the suspension on a slide, which has been cleaned with alcohol to remove most of the grease. With a needle or other pointed instrument spread the drop out into a thin film.

3. Dry on a flat surface over a boiling water bath.

4. Without removing from the water bath, cover with the following staining fluid for 1 minute:

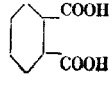
Rose bengal (dye content about 80%)	1	g.
$CaCl_2$	0.01	g.
5% aqu. sol. phenol	100	cc.

5. Wash rapidly in tap water.

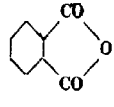
6. Dry; and examine under microscope. A rather high power is necessary, the combination of lenses giving best results of any that have been tried being a 1.9 mm. fluorite objective with a 12.5 compensating or planoscopic ocular.

4. PHENOLPHTHALEIN AND THE SULFONPHTHALEINS

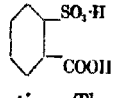
A phthalein is a compound of phthalic acid:



or rather of phthalic anhydride:



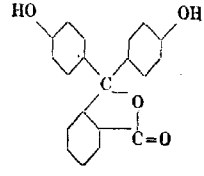
with phenol or a phenol derivative. If phthalic acid is heated with phenol and sulfuric acid it combines with two molecules of the latter and forms phenolphthalein. In the same way, a sulfonphthalein is a compound of ortho-sulfo-benzoic acid:



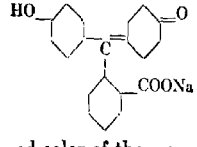
and phenol or a phenol derivative. These compounds, altho sometimes behaving as dyes, are not used as dyes or stains, but as in-

dicators. For this purpose the members of the group are very valuable.

Phenolphthalein, altho not used as a dye, is colored and is apparently capable of salt formation. In acid solutions it is colorless, and is assumed to have the formula:



Upon neutralization the alkali is believed to attach itself to the CO-group, which breaks the five-sided ring (the lactone ring) and causes one of the benzene rings to take on quinoid form, thus:



With this change, the red color of the compound appears, but disappears again if the solution is made acid so as to destroy the quinoid structure. This makes the compound a very valuable indicator.

The sulfonphthaleins have no use in practical dyeing, altho they are weakly acid dyes. Their real value is as indicators. Quite a long series of them has been prepared, which in general show their deepest color in alkaline solutions and turn yellow on the addition of acid. Some of them, such as thymol-sulfonphthalein (thymol blue), show two colors besides yellow, one in strong acid solutions and the other in strong basic solutions, while in solutions near the neutral point they are yellow. That these color changes are due to alterations in the structure of the molecule, such as the disappearance and reappearance of the quinoid ring, is generally assumed; but in the case of these compounds the relation of structure to color is complicated and has not yet been worked out to general satisfaction.

The colorimetric measurement of H-ion concentration depends upon these color changes. The color change of an indicator takes place within a short range on each side of that point in the H-ion scale at which the dye is 50% dissociated, i. e., occurs half in the form of the undissociated dye and half in the form of free ions. This point on the pH-scale (at which an indicator is 50% dissociated) is very important, and corresponds to the invert logarithm of its dissociation constant. The dissociation constant of

an indicator, in other words, is a quantity whose invert logarithm is the pH-value of the point at which the indicator is half dissociated. The approximate invert logarithm of the apparent dissociation constant is denoted by chemists by the symbol pKa; the value of this quantity is given in the case of each of the indicators listed below.

For a short distance on each side of the dissociation constant, every shade of the indicator corresponds to a definite pH-value; this zone is known as the sensitive range of the indicator. Roughly speaking, the sensitive range of any indicator extends for about 0.8 pH to each side of its pKa-value. Thruout its sensitive range, an indicator can be used to determine the H-ion concentration of a solution by comparing its shade with that produced in standards of known reaction. The various indicators of this group differ in their strengths as acids (i. e. the extent to which they dissociate); and the greater their strength the lower the pH-value of the point at which they are 50% dissociated. It is plain, therefore that different indicators may be selected according to their dissociation constants to cover successive portions of the H-ion scale. Fortunately nearly all of them have different dissociation constants; so that by employing a series of them one may determine the H-ion concentration of solutions of any reaction ordinarily encountered.

There are two general types of indicators, those which show only one color in the useful range, and those which change from one color to another. The phthaleins, such as phenolphthalein, change from colorless substances in their acid ranges to colored compounds in their alkaline ranges. The sulfonphthaleins, on the other hand, change from yellow to some deeper color. With the first type of indicator the H-ion concentration may be determined by the alteration in intensity of color, with the second type by alteration in hue. The two-color indicators, such as the sulfonphthaleins, are more satisfactory because one can measure changes in hue more accurately than changes in color intensity, especially when one must depend on rather crude methods of colorimetry as is usually the case when indicators are employed.

The sulfonphthalein indicators are especially valuable as indicators for still other reasons. Unlike the azo compounds, such as methyl red, they are very stable chemically, while they are less affected by the presence of neutral salts and proteins than are many other indicators.

The first list of sulfonphthalein indicators was published by Clark and Lubs (1917), who to make their series complete had to include methyl red, an azo dye, less satisfactory because its solutions are subject to reduction on standing. More recently Cohen (1923, 1926) has added six new sulfonphthalein indicators, among them two (brom cresol green and chlor cresol green) which have sensitive ranges so close to methyl red as to make the latter unnecessary.

The only staining application of this series of compounds seems to be as vital dyes. In this use their value depends upon their indicator properties, as they are employed in studying the reaction of tissues or body fluids. They are also valuable indicators in micro-injection of cells.

These indicators, unlike the usual dyes, are ordinarily furnished in the form of color acids, which are practically insoluble in water. They must accordingly be employed in alcoholic solution or must be converted into the di-sodium salt with the proper amount of NaOH, the quantity of the latter to use being figured by the following formula:

$$\text{Weight NaOH} = \frac{\text{Weight indicator} \times 40}{\text{Molecular weight indicator}}.$$

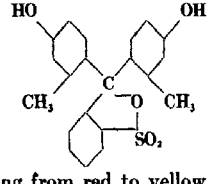
The alcoholic solutions are simpler to prepare and for ordinary indicator purposes are equally satisfactory; they cannot, of course, be employed, in vital staining.

In the following list the compounds are arranged in the order of their dissociation constants and hence in that of their sensitive ranges in the pH-scale.

META-CRESOL PURPLE

pKa = 1.5, 8.3

This indicator is *m*-cresol sulfonphthalein.

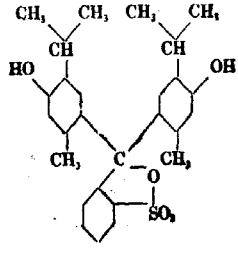


An indicator changing from red to yellow in its acid range and from yellow to purple in its alkaline range.

THYMOL BLUE

pKa = 1.5, 8.9

This is thymol sulfonphthalein:

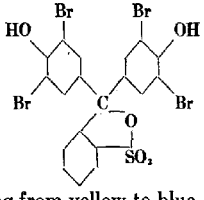


Thymol blue is now a well known indicator, used both in its acid range (red to yellow) and in its alkaline range (yellow to blue). In the latter range it may well be replaced with meta-cresol purple.

BROM PHENOL BLUE

pKa = 4.1

This is tetra-brom-phenol sulfonphthalein.

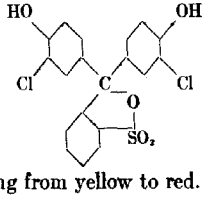


An indicator changing from yellow to blue.

CHLOR PHENOL RED

pKa = 6.0

This is di-chlor-phenol sulfonphthalein.

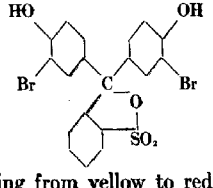


An indicator changing from yellow to red.

BROM PHENOL RED

pKa = 6.2

This is di-brom-phenol sulfonphthalein.

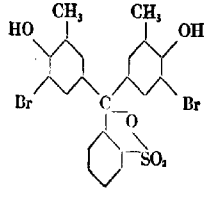


An indicator changing from yellow to red thru a range almost identical with that of the better known brom cresol purple. It is recommended in place of the latter because it is free from troublesome dichromatism.

BROM CRESOL PURPLE

pKa = 6.3

This is di-brom-ortho-cresol sulfonphthalein:

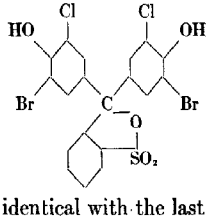


A well known indicator changing from yellow to purple. It is now coming to be replaced with brom phenol red.

BROM CHLOR PHENOL BLUE

pKa = 4.0

This is di-brom-di-chlor-phenol sulfonphthalein.

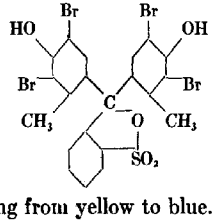


An indicator almost identical with the last in range, and showing the same change in color.

BROM CRESOL GREEN

pKa = 4.7

This is tetra-brom-meta-cresol sulfonphthalein:

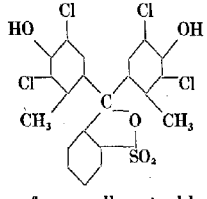


An indicator changing from yellow to blue.

CHLOR CRESOL GREEN

pKa = 4.8

This is tetra-chlor-meta-cresol sulfonphthalein.

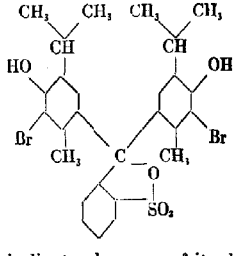


An indicator changing from yellow to blue thru a range only a little more acid than that of methyl red.

BROM THYMOL BLUE

pKa = 7.1

This is di-brom-thymol sulfonphthalein:

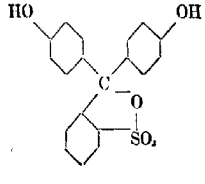


It is a very valuable indicator because of its dissociation constant at close to true neutrality. It is yellow in acid, green in neutral, and blue in alkaline solutions.

PHENOL RED

pKa = 8.2

This is phenol-sulfonphthalein:

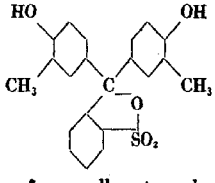


An indicator changing from yellow to red, employed for some time in physiological work, and well known before the paper by Clark and Lubs above mentioned.

CRESOL RED

pKa = 8.3

This is ortho-cresol-sulfonphthalein:



An indicator changing from yellow to red.

PHENOLPHTHALEIN

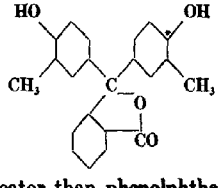
pKa = 9.4

This indicator, of which the formula is given on p. 134, is quite useful in the alkaline range centering around pH 9.4, with a color change from red in alkaline solutions to colorless in acid ones. It has the disadvantage of a one-color indicator discussed on p. 135.

CRESOLPHTHALEIN

pKa = 9.7

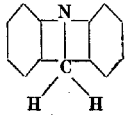
This indicator is closely related to phenolphthalein, having the constitution:



It is a newer indicator than phenolphthalein, and is generally regarded as preferable when used in conjunction with the sulfonphthaleins, because its range does not overlap quite so far the alkaline range of thymol blue. It is a one-color indicator and its color change is the same as that of phenolphthalein.

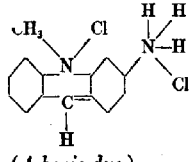
ACRIDINE DYES

There is a small group of dyes derived from the compound acridine



which is closely related to xanthene. They are not xanthene dyes, but are included in this chapter for convenience. There is only one of interest to the biologist.

ACRIFLAVINE C. I. NO. 790



(*A basic dye.*)

This yellow dye is one of those developed by Ehrlich for its therapeutic value. It is marketed at present as a disinfectant. It is employed as a bacteriostatic agent by Churchman mixed with methyl and crystal violets under the name of "acri-violet."

CHAPTER VIII

COMPOUND DYES

THERE are two ways in which dyes may be compounded. In the first place it is possible to mix mechanically any two dyes, and if they are of different colors with different selective powers, double staining effects may be procured. In the second place, it is often possible to form a chemical union between two dyes and thus to obtain an entirely new compound which may have quite striking staining properties. It is such compounds as these, rather than simple mechanical mixtures, that are ordinarily referred to as compound dyes.

The simple anilin dyes, it will be recalled (see Chapter II), owe their properties as dyes or as biological stains to the basic or acidic character of the dye molecule. Those parts of the protoplasm which are acid in nature (e. g., chromatin) tend to react with the basic dyes and to be colored by them; while those which are basic (e. g., cytoplasm) react similarly with the acid dyes. (This, to be sure, is not the whole theory of staining, as the process is quite complex and involves physical and mechanical factors as well; but it serves to illustrate the difference between the two kinds of stains.) Now, as already explained, the dyes are not used as free acids or free bases; but rather as sodium or potassium salts of the acid dyes, and as chlorides (or salts of some other colorless acid) of the basic dyes.

It is well known that when two salts, such as sodium chloride and ammonium nitrate, are mixed in solution there is an interchange of ions (metathesis), and the resulting solution, when it reaches equilibrium, contains not only the original salts but also the four free ions and the two alternate compounds as well, in this case sodium nitrate and ammonium chloride. Now if one of these two new compounds happens to be insoluble, as silver chloride for example, which would have been formed if silver nitrate had been substituted for ammonium nitrate, it is thrown out of solution, and equilibrium is not reached until the solution is free (or at least practically free) from the two ions which are insoluble in combination. In the same way, when a sodium salt of an acid dye and a chloride of a basic dye are mixed in solution, there is a similar tendency for the ions to interchange. Ordinarily the dyes are weaker acids and bases than the chlorine and sodium ions respectively; and if the compound dye formed were soluble in water there would be little chance for much of it to be produced. As a matter of fact, however, it is generally insoluble and is therefore thrown out of solution; hence the compound dye can be formed in considerable quantity.

Now compound dyes of this sort are sometimes referred to as neutral dyes or neutral stains. This terminology, of course, does not indicate that they are neutral in reaction any more than do the corresponding terms acid and basic dyes. A dye chemist, in fact, uses the term neutral dye in an entirely different sense; but it is frequently employed by biologists, especially by Ehrlich, to refer to the basic dye salts of dye acids. In this chapter these compound dyes will be called *neutral stains*, as this latter term is not employed by dyers or dye chemists in any sense, and is unlikely, therefore, to be misunderstood. Ehrlich also uses another term to apply to some of these compounds, namely "tri-acid dyes." He uses this term on the assumption that, while in the ordinary basic dyes only one of the three affinities of the dye for acid is satisfied, it is possible to satisfy all three and in this way to saturate the basic dye with acid. This assumption of his seems to be incorrect; but Ehrlich's "tri-acid stain" (see below) is so well-known that an explanation of the term is necessary.

It is possible to obtain an endless variety of such dyes; but in practice only a certain number of them have proved useful. Among the basic dyes the most suitable for this purpose are methylene blue and the rosanilins (which act as strong ammonium bases); among the acid dyes, eosin and the sulfonic acids (e.g., orange G and acid fuchsin).

Altho the neutral stains are insoluble in water, they are soluble to a greater or less extent in excess of either the acid or the basic dye. Thus if a watery solution of acid fuchsin is neutralized by adding drop by drop a watery solution of methyl green, there is at first no precipitation, because the methyl green salt of acid fuchsin is kept in solution by the excess of acid fuchsin. After the proper amount of methyl green has been added, however, and the mixture has stood long enough for the reaction to take place, the neutral stain is precipitated and the solution becomes nearly colorless. Then if more methyl green is added the neutral stain is slowly dissolved again; but as a rule neutral stains are less soluble in excess of base than in excess of acid.

As simple aqueous solutions of these compound dyes are impossible and as alcoholic solutions of dyes do not stain well, various methods are employed to secure their action on the tissues. In some instances they are kept dissolved by the presence of an excess of acid or base (particularly the former); in others a certain quantity of acetone or methylal is used to hold the neutral stain in solution; sometimes (as in the original Romanovsky stain) the compound dye is used immediately after mixing, before the reaction is complete or precipitation has taken place; or again (as in the Wright stain) methyl or ethyl alcohol may be used as a solvent, and then after applying the alcoholic solution to the slide it may be diluted with water. This latter method is particularly effica-

ceous, because the dissociation which takes place upon the addition of water causes the production of various dye compounds which may stain intensively and very selectively.

It is assumed that these compound dyes act on the protoplasm somewhat as follows: certain parts of the cell have an affinity for the neutral stain and take it up as such; others, having an affinity for the basic dye, break up the neutral stain so as to obtain the basic portion of it, or if dissociation has taken place, take up the basic ion directly; while other parts of the cell with an affinity for acid dyes similarly combine with the acid portion of the stain. These three types of cell structures are known as neutrophile, basophile and oxyphile elements, respectively. The differentiation thus produced gives the neutral dyes their great value.

Ehrlich's "Triacid Stain."

The first neutral stain proposed for microscopic work was the "triacid stain" of Ehrlich (1910, II, 313). In forming this compound dye, acid fuchsin and orange G are mixed in solution and to the mixture is then added such a quantity of methyl green that there is still an excess of the acid dye. This excess of the acid dye allows the neutral stain to stay in solution. The dye thus formed is a very valuable blood stain, and brings out finely the different structures in the leucocytes.

Slight modifications of this stain have been used for tissues. The best known of these modifications is that of Biondi-Heidenhain.

Eosin-Methylene-blue Compounds*

The first worker to combine eosin and methylene blue was Romanovsky (1891). He realized that a mixture of these two dyes had great selective properties as a stain, and showed it to be excellent for blood, particularly in bringing out the malarial parasite. He also appreciated that it was more than a mere mixture of the two dyes and that some new dye having the property of giving the nuclei a red color was present. It was some time later before the nature of this new dye was known, altho it was subsequently named azure I or methylene azure; its true chemistry has scarcely been understood until very recently (see p. 61). Methylene violet, which probably was also present, had already been described by Bernthsen (1885). How these new dyes were formed in the Romanovsky stain was not known then; altho Romanovsky stated that different lots of methylene blue solution varied in their ability to give a good blood stain, and that old solutions on which a scum had formed were best.

Present day blood stains are often spoken of as modified Romanovsky stains; altho the modifications are so great as to make them

*A good account of the history of these blood stains is given by MacNeal (1906).

of a very different nature. The first modification was made by Nocht (1898) who concluded that the differential staining was due to the formation of other dyes by the decomposition of methylene blue. Unna (1891) had already described what he called polychrome methylene blue, made by heating a solution of methylene blue on a water bath with potassium carbonate. Nocht decided to use this in the Romanovksy stain instead of untreated methylene blue. He found that it gave very good results if properly neutralized before mixing with eosin; and then learned that better results could be obtained by the use of a smaller amount of alkali and a longer period of polychroming, without subsequent neutralization.

The next step in preparing blood stains was made by Jenner (1899) who collected the precipitate formed when methylene blue and eosin are mixed, and redissolved it in methyl alcohol. He did not use polychrome methylene blue, and his stain lacked the nuclear staining principle of Romanovsky's and Nocht's stains; but it was an important step in that he showed the possibility of collecting the precipitated compound stain and of dissolving it in some solvent other than water. Combining this procedure with the Nocht stain was the next logical step and was taken independently by Reuter (1901) and by Leishman (1901). The method thus introduced was briefly to follow Nocht's technic of combining eosin with polychrome methylene blue, but then to filter off the precipitate and to redissolve it in methyl alcohol, not adding further water until the moment of applying the stain to the blood films.

Modern blood stains are in general modifications of Leishman's, differing only in detail. Wright's modification, the one most used in America, (see Mallory and Wright, 1924, p. 170) differs from Leishman's only in that he prepared polychrome methylene blue by heating for only an hour in flowing steam, whereas the Leishman technic calls for twelve hours at 65°C, with subsequent standing for ten days. Balch's modification calls for a polychrome methylene blue prepared by standing ten days with precipitated silver oxide.

Giemsa's and MacNeal's modifications are somewhat different. Giemsa obtained an oxidation product of methylene blue which he named azure I and considered a pure dye; this he combined with eosin in order to obtain a more definite compound than when polychrome methylene blue is used. Then to obtain better differentiation he mixed it with methylene blue. Following his instructions, the Grübler Co. put on the market a product known as azure II, which was a mixture of azure I and methylene blue in equal parts; and also a compound known as azure-II-eosin, which was an eosinate of azure II, or more precisely a mixture of the eosinate of methylene blue with that of azure I, in equal parts. This latter compound is generally known as the Giemsa stain.

MacNeal (1922) has proposed a method for obtaining a very similar blood stain, called the tetrachrome stain, prepared on different principles. MacNeal appreciated that polychrome methylene blue is a very indefinite mixture of oxidation products of that dye, prominent among them being methylene violet (Bernthsen) and methylene azure. He showed that by mixing these two dyes, with methylene blue and eosin, dissolving in methyl alcohol, and employing like a Leishman or Wright stain, one could obtain results almost identical with those secured with one of these other blood stains without the uncertainties introduced by the polychroming of the methylene blue. More recently MacNeal (1925) has recognized the various azures present in oxidized methylene blue (see pp. 61–67) and has shown that azure A is the most satisfactory of these in the tetrachrome stain. Azure A is now available commercially and is to be recommended for use in this stain. The tetrachrome stain, when purchased today in prepared form, contains in most instances azure A instead of the less definite azure I of Giemsa. It will readily be seen that the dry tetrachrome stain is a mixture rather than a chemical compound like the Leishman stain and its modifications. The four dyes mixed together combine but very slowly even after dissolving in the alcohol; and the first pronounced chemical reaction occurs only after diluting with water in actual application. There is, in fact, evidence that the presence of the compound dyes, while still in the alcoholic solution is harmful, and that the poor keeping qualities of the tetrachrome solution frequently observed are due to the slow formation of such compound dyes even while in alcoholic solution. It has been found, however (Conn 1927b), that a solution of the tetrachrome stain may be kept almost indefinitely if properly prepared. The method is to keep the alcoholic solution at about 50° C for about 48 hours to allow the reaction that occurs to become complete, and then to filter; the filtered solution may be kept for months.

Some difficulty was experienced at first in compounding the eosin-methylene-blue blood stains when imported dyes were no longer available. In some cases these difficulties were probably due to poor methylene blue or to poor eosin; but upon investigation the solvent, rather than the dyes themselves, has been found to be most often at fault. As stated above, the precipitated compound dye must be dissolved in methyl alcohol; but there are many grades of methyl alcohol and not all are equally suitable for the purpose. Apparently absolute purity is not needed; but two points are very important; the methyl alcohol must be neutral in reaction, and it must be free from acetone. In specifying a methyl alcohol for the blood stains, these two properties should be insisted upon. Very good methyl alcohol for this special purpose is now on the market.

The three blood stains for which there is now most demand in America are Jenner's stain, Wright's stain and the tetrachrome stain, Wright's apparently being the most popular. Jenner's stain, prepared from unpolychromed methylene blue is much the least satisfactory of these three, and it is difficult to see how anyone familiar with either of the others would choose it. Wright's stain and the tetrachrome stain both give almost identical results and are handled very similarly. If one is to prepare the stain himself, there are reasons for preferring the tetrachrome stain as it can be made up with more certainty of obtaining a satisfactory product. Ordinarily today, however, one purchases his blood stains already prepared by some manufacturer; and the Wright's stain on the market seems to be as reliable as the tetrachrome stain. All three of the blood stains just named have been put on the certification basis by the Commission; and if sold under the Commission label, a dye must have given good results in the Commission laboratory.

Wright's Stain:

0.5% aqu. sol. sodium bicarbonate.....100 cc.
Methylene blue, medicinal, (dye content about 90%).....0.9 g.

Heat in steam sterilizer at 100°C one hour, in containers in which the solution is not over 6 cm. deep. Filter after cooling. Add the following solution:

Eosin Y (dye content 85%).....1.0 g.
Distilled water.....500 cc.

Mix thoroly and collect the abundant precipitate on a filter, and dry. If the two dyes are combined in the proportions indicated by the quantities and dye contents above specified, the maximum yield of methylene blue eosinate should be obtained. This proportion may be changed without altering the nature of the compound dye secured, but the excess of either methylene blue or eosin would be wasted. (This stain, either dry or dissolved in methyl alcohol, may be purchased from stain companies at present, usually more uniform in quality than can be made up by the user.)

Dissolve the dried precipitate in the following proportions:

Wright's stain (dry).....0.1 g.
Methyl alcohol, absolute, neutral, acetone-free.....60 cc.

Allow solution to stand a day or two; then filter. Always filter when using.

To use the stain place about 10 drops of the alcoholic solution on the slide or cover glass bearing the blood film, allowing it to stand about one minute. Then add about 10 drops of distilled water drop by drop; or preferably a phosphate buffer solution, adjusted to about pH 6.5. After standing two minutes, pour off stain and wash with water (or preferably the above mentioned buffer solution) until the thin portions of the stained film are pink. Dry by blotting carefully.

Tetrachrome stain:

Methylene blue, medicinal, (dye content about 90%).....1.0 g.
Azure A.....0.6 g.
Methylene violet, Bernthsen, free base.....0.2 g.
Eosin Y, (dye content 80–85%).....1.0 g.

(These dyes, mixed in the proper proportions may be purchased from stain companies, either dry or in solution, under the name of tetrachrome stain, MacNeal.)

Dissolve 0.5 g. of the dry ingredients in 100 cc. methyl alcohol, neutral, acetone free by heating to 50°C. Shake thoroly and leave at 37°C for one or two days, with occasional shaking. Filter off any precipitate that forms. If prepared according to directions the solution should keep fairly permanently; if the above method of dissolving is not carefully followed, however, the solution is likely to deteriorate rapidly.

Employ on blood films exactly as in case of Wright's stain.

Other Compound Stains

Various other compounds of acid and basic dyes have been used for special purposes. The basic dyes employed in these compounds most commonly are perhaps methyl green and methylene blue; but sometimes basic fuchsin, pyronin or rhodamine or even a weak base like neutral red is used. Most common among the acid dyes in these compounds are eosin, orange G and acid fuchsin; but certain others are occasionally employed. Picric acid forms a few useful compound dyes, rosanilin picrate (i. e. the compound of basic fuchsin and picric acid) being especially well known as a tissue stain.

The Pappenheim panoptic triacid stain is a modification of Ehrlich's triacid compound. In this combination methylene blue or methylene azure is substituted for methyl green. It is a tissue stain of use in certain special technics. Another well known neutral stain is the Twort (1924a and b) formula in which neutral red and light green are combined. The Twort stain is valuable for staining animal parasites and other animal organisms in tissues. Maurer and Lewis (1922) (see page 110) have combined safranin in a neutral stain with some acid violet and have employed the compound dye in staining glandular tissue.

Ehrlich has proposed various other neutral stains, the best known being a compound of acid fuchsin and methylene blue used for staining blood; and a compound of narcein, an acid dye, with two basic dyes pryonin and methyl green or methylene blue.

CHAPTER IX

THE NATURAL DYES

AS STATED above (p. 16) the group of natural dyes is shrinking as more and more of them are being produced by artificial means. Alizarin, for example, in the form of madder, used to be extracted from the roots of *Rubia tinctorum;* but the artificial manufacture of this dye is now much more economical. The group of natural dyes, as ordinarily recognized, contains only those which are not yet produced by artificial means. Indigo, however, is listed in this chapter, because in its chemistry it does not fall in well with any of the groups of artificial dyes. It is still obtained in part, moreover, from the indgo plant, altho under present-day conditions its artificial manufacture is ordinarily the more economical.

The chemistry of the natural dyes is less definitely known than that of the artificial dyes. This is easily understood; for it will be recalled that there are two ways of obtaining information as to the chemistry of unknown compounds: the first by decomposing them into simpler compounds of known composition; and the second by manufacturing them from known compounds. In the case of dyes not yet prepared artificially the second of these two lines of procedure is out of the question; hence the difficulty in learning their exact chemical structure.

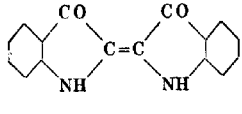
The most important natural dyes for the biologist are hematoxylin, indigo, cochineal (and its derivatives), orcein, and litmus.

The Indigo Group

INDIGO C. I. NO. 1177

Synonym: *Indigo blue.*

The plants from which indigo was formerly exclusively manufactured are largely species of the genus known as Indigofera, altho some indigo-bearing plants are recognized by botanists as belonging to different genera. In these plants is a glucoside, indican, which is converted by fermentation into the dye indigo. Various formulae have been given for indigo; the one favored at present is based upon its method of artificial manufacture:

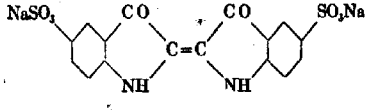


In this formula the exact chromophore group is uncertain; but the ketone group (CO) in a closed ring occurs so often in dyes that it is regarded as probably having chromophoric properties.

INDIGO-CARMIN C. I. NO. 1180

Synonym: *Indigotine Ia.*

This is the sodium salt of indigodisulfonic acid:



Indigo carmin is a blue dye of acid properties, which is sometimes used as a plasma stain in contrast to carmin, either mixed with it or following it.

COCHINEAL PRODUCTS C. I. NO. 1239

Cochineal is a dye that has long been well known. It is obtained from a tropical insect generally known as the cochineal insect. By grinding and extracting the dried bodies of the female of the species in question a deep red dye is obtained, which is known as cochineal. On treatment with alum this solution yields a product somewhat more free from extraneous matter, known as carmin. This is the form in which the dye is generally obtained by the microscopist. Cochineal products are used in various ways in microscopic technic, generally as nuclear dyes. They are extremely valuable in cases where it is desirable to stain in bulk before sectioning.

Cochineal, itself, has been used for various purposes in microscopic technic, even tho less used today than carmin. Alone it has little value, to be sure, for it has no direct affinity for tissues unless they contain iron, aluminium or some other metal. It is most commonly employed either with or following a salt of one of these metals as a mordant. A tincture of cochineal, that is an alcoholic solution containing calcium and aluminium chlorides, has been used by Mayer both on sections and for staining in bulk; but its most common method of use is with alum in watery solution. An alum-cochineal of this sort was first used independently by Mayer, Czokor, and Partsch; it can be used for sections, and is specially recommended for staining in bulk, by which technic it stains nuclei violet red, and blood and muscle cells orange, while the cytoplasm is but weakly colored. A chrom-alum-cochineal has been used by Hansen for staining sections. Spuler recommends an iron-alum-cochineal for staining in bulk when the sections are to be photographed, the technic bringing out nuclei, the blood in the tissues,

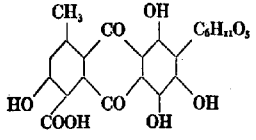
and the muscle striations; sections may also be stained by the same method. By this technic the iron alum is applied first to the tissues as a mordant, and then followed by the stain. In Hansen's ferri-cochineal, on the other hand, the iron alum is mixed with the dye, and the mixture used for staining sections of tissue.*

Carmin. Carmin is of considerable historic interest. It was used as early as 1770 by Hill and in 1839 by Ehrenberg, altho as we have seen (p. 9) not exactly for histological purposes. It was also employed in 1849 by Göppert and Cohn, by Corti in 1851, and by Hartig in 1854–8, these being the first uses of dyes in histology. It is still a valuable stain today, in spite of the enormous variety of synthetic dyes now available. It is much used for staining in bulk, particularly in embryological work. A well known formula is Schneider's aceto-carmin, which is a valuable chromatin stain for fresh material in smear preparations. Belling's iron aceto-carmin is valuable for staining chromosomes in smear preparations from anthers. Alum carmin was used by Grenacher for similar purposes. Carmin is only slightly soluble in water at a neutral reaction; so solutions must be either acid (like the three above) or alkaline. Three alkaline formulae are of considerable use: ammonia carmin, which has been used both for injection and for staining sections; soda carmin, used primarily for injection; and Mayer's magnesia carmin, useful either for sections or for staining in bulk. Alcoholic solutions are also used: Grenacher's borax carmin (or as modified by Mayer) being a splendid nuclear stain for sections; and the hydrochloric carmin of Mayer serving both for sections and for staining in bulk. A special formula containing aluminium chloride (known as mucicarmin) has been proposed by Mayer and is used for staining mucin. In double staining it is sometimes used with indigo carmin; but most often with picric acid or spirit blue. Picro-carmin is a very well known combination used for double staining effects in sections, particularly for nervous tissue; it stains nuclei red and cytoplasm yellow.

One of the most recent and important uses of carmin is in Best's carmin stain for glycogen. The method is simple and the result beautiful, the red glycogen standing out in sharp contrast to the blue of the nuclei after staining in alum hematoxylin. The stain is permanent; the method is of much importance both to the pathologist and to the histologist.

Carminic acid. The dye principle of carmin and cochineal is carminic acid. This product is obtained by extracting the insect bodies with boiling water, treating the extract with lead acetate or barium hydrate, and then decomposing the lead or barium carminate with sulfuric acid. The exact composition of carminic acid is still somewhat uncertain; so far as known, it is:

*For literature references to the procedures listed in this chapter see pp. 174 to 195 and 204 to 216.



It is a fairly strong dibasic acid and forms readily soluble salts with the alkali metals, and insoluble salts with the heavy metals. Aluminium carminate (obtained by precipitation from aluminium acetate and carminic acid or ammonium carminate) is soluble in aqueous or weak alcoholic solutions of acids.

A slightly different aluminium compound, formed by mixing alum and carminic acid is used in histology. This combination was called carmalum by Mayer, and has also been used by Grenacher and Rawitz; it is a useful nuclear stain for sections, and is often employed with light green or indigo carmin as a contrast stain. A so-called muci-carmin, an acid solution containing aluminium chloride, has been employed by Rawitz to stain mucin; while Mayer's para-carmin, containing aluminium and calcium chlorides, is used both for sections and for staining in bulk. By others a combination of iron with carminic acid has been used for similar purposes.

Carmein. Carmin, kept in ammoniacal solution, changes in its properties, due to oxidation. The oxidized carmin, often known as carmein, can be obtained by treating a carmin solution with hydrogen peroxide and precipitating with alcohol. It is a dark colored mass which can be ground into a black powder.

Orth's lithium carmin: Orth's original papers do not give any definite formula for this staining fluid. The following is taken from Krause (1926 I, p. 265).

Sat. aqu. sol. lithium carbonate (about 1%) ..... 100 cc.
Carmin ..... 2.5–5 g.

Boil 10–15 minutes.

Sections are stained 2–5 minutes, then differentiated in 0.5–1% HCl-alcohol, and washed in water.

Mayer's "carmalum":

Carminic acid ..... 1 g.
Alum ..... 10 g.
Distilled water ..... 200 cc.

Dissolve by heating, pour off clear, or filter. This solution remains clear if an antiseptic is added (for example, a few crystals of thymol or 0.10% salicylic acid).

Grenacher's borax carmin:

Carmin ..... 2–3 g.
4% aqu. sol. borax ..... 100 cc.

Dissolve by boiling half an hour. When cool add an equal quantity of 70% alcohol. Allow to stand a few days to a few weeks, with occasional shaking, and filter.

Mayer modifies this by making up in 50–70% alcohol direct, which dissolves 1% or less of borax and about 1% of carmin.

This solution is recommended for staining in bulk. After staining, material is brought into 70% alcohol acidulated with 5 drops of hydrochloric acid to 100 cc. of the alcohol; after clearing wash with neutral 70% alcohol.

Best's carmin stain for glycogen:

Carmin	2 parts
K_2CO_3	1 "
KCl	5 "
Water	60 "

Boil a few minutes, but do not over-heat. After cooling, add 20 parts ammonium hydrate. May be kept in tightly stoppered bottles a few months. Filter before use.

This staining fluid is to be used on material fixed in Carnoy's acetic alcohol or in absolute alcohol alone embedded in celloidin, or treated with celloidin after paraffin embedding. One should first stain with hematoxylin or hemalum, then differentiate with HCl-alcohol. For use mix 2 parts of the above solution with 3 parts ammonia and 3 parts methyl alcohol. Differentiate in a mixture of: absolute* alcohol 80 parts, methyl alcohol 40 parts, distilled water 100 parts. The glycogen stains a bright red.

Belling's iron aceto-carmin:

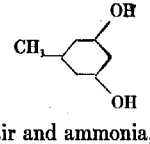
Prepare ordinary aceto-carmin (Schneider's) by saturating boiling 45% acetic acid with carmin, then filtering.

To this add a few drops of ferric hydrate dissolved in 45% acetic acid until the liquid becomes bluish red, but without visible precipitate. Then add an equal amount of ordinary aceto-carmin.

This staining fluid is used primarily for staining chromosomes in smears of anthers.

Orcein and Litmus C. I. No. 1242

Both orcein and litmus are obtained from certain lichens, *Lecanora tinctoria* and *Rocella tinctoria*. These lichens are colorless, but when treated with ammonia and exposed to the air, blue or violet colors develop. The colors are due to certain acids, one of which is orcin:



Orcin, acted upon by air and ammonia, becomes orcein.

ORCEIN

The exact formula of orcein is unknown. It is a weak acid, soluble in alkalies, with a violet color.

*Considering the quantity of water in this mixture, one may employ 95% alcohol with practically identical results.

Unna has used orcein in alcoholic solution for staining elastin tissue; he has employed it for connective tissue, following polychrome methylene blue; and for plasma fibrils in the epithelium, following anilin blue; also with anilin blue or acid fuchsin in studying the process known by him as chromolysis. It has found less frequent use among other histologists; but has been employed by Israel in acetic acid solution for staining sections (nuclei staining blue, cytoplasm red); and by Moll, dissolved in weak hydrochloric acid, for staining sections of embryos.

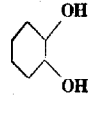
LITMUS

The exact composition of litmus is likewise unknown. It is obtained from the same lichens as orcein, treating them with lime and potash or soda, in addition to air and ammonia. Its colored principle is known as azolitmin.

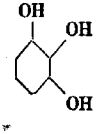
Litmus scarcely needs comment here. It is a feeble dye and is never used as an histological stain. Its classic use is for indicator purposes; but it is now coming to be largely replaced by the various synthetic dyes (especially sulfonphthaleins) which change color thru an hydrogen-ion range near the neutral point.

Brazilin and Hematoxylin

The two natural dyes, hematoxylin and brazilin are closely related chemically and upon decomposition yield the two compounds, pyrocatechin



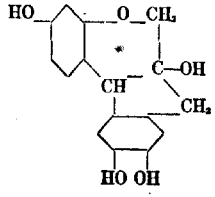
and pyrogallic acid



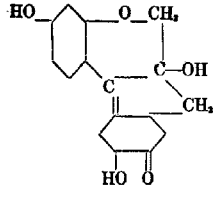
Both dyes are obtained by extraction of the bark of certain trees, hematoxylin from logwood and brazilin from brazil wood (red wood). Both trees are legumes and belong to the family Cesalpiniaceae; they are found only in the tropics. Hematoxylin comes from a single species; while brazil wood is a term applied to various different species all yielding brazilin.

BRAZILIN C. I. NO. 1243

The composition of this substance is supposed to be:



Its solution is colorless, but it becomes red on exposure to the air, as it is then oxidized into the dye brazilein, which probably has the formula:



With alum it is employed as a nuclear stain (known as brazalum) by Mayer. It is also used by Hickson for similar purposes following treatment with iron alum as a mordant. Recently Belling proposed its use in place of carmin for staining plant chromosomes in fixed or fresh material.

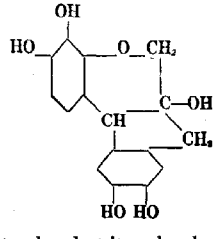
HEMATOXYLIN C. I. NO. 1240

Hematoxylin, as is well known, is a constituent of logwood, a product of South America. It was first obtained about 1840 by extracting logwood chips with ether, evaporating, digesting with alcohol and standing with water after distilling off the alcohol; hematoxylin was found to separate out in crystals. A little later a similar method was employed, but instead of using logwood chips, the starting point of the process was with commercial logwood extract, which is the dried aqueous extract of the wood. In the present method of manufacture the dried commercial extract is extracted with ether in a continuous extraction apparatus, evaporated to dryness, dissolved in water, filtered and crystallized out of the solution. All of these steps, particularly the ether extraction, are slow and difficult to handle on a factory scale, requiring special expensive apparatus. Hence most of the hematoxylin now sold in the United States is the product of a single manufacturer, altho not always sold under the label of this company.

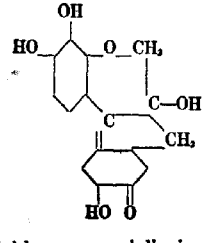
Two recent papers, by McClung (1923) and Conn (1927c) respectively, have discussed the present hematoxylin situation so

far as concerns the availability of a satisfactory product in America. They can be summed up briefly here by saying that the first American hematoxylins, marketed during the war, were very crude and proved quite unsatisfactory, but that a constant improvement in the supply has been made by the manufacturers since then. The first American product was dark in color; and there was so much demand for a light colored hematoxylin that the manufacturers introduced the process of bleaching with sulfur dioxide. In cooperation with the Stain Commission it was subsequently found that this bleaching injured the staining qualities, and the manufacturers changed their method and began turning out a darker colored but more satisfactory stain. There were still some complaints of the keeping qualities of the solutions made from this hematoxylin; and as a result another change in manufacture was made in 1926. The work has progressed slowly, because when the question of keeping qualities is involved testing is time-consuming; but it can now safely be said that an American hematoxylin is available which is more satisfactory than the product in use before the war.

Hematoxylin is a homolog of brazilin, having one more hydroxyl group, the generally accepted formula being:



Like brazilin, it is not a dye, but its color develops in solution upon standing, due to the oxidation into hematein, which is homologous to brazilein and probably has the formula:



Hematein is available commercially in dry form. Less attention has been given to its production than to that of hematoxylin. Hence it frequently results that a hematein sold by some company

is more crude than their hematoxylin, altho theoretically hematein is a derivative of hematoxylin. At the time this is written no domestic source of hematein is known as satisfactory as the imported product; but it is probable that within a year, or an even shorter period, the situation will be changed.

Hematoxylin is without question one of the most important biological stains. It is as valuable to the cytologist and histologist as methylene blue is to the bacteriologist; and probably is second only to methylene blue in the number of different purposes for which it is used. It is valuable not only because it is a powerful nuclear stain and a chromatin stain *par excellence*, but also because it has striking polychrome properties. With the proper differentiation it is possible to get several shades intermediate between blue and red to show in the same preparation.

Hematoxylin is seldom used alone, as it has little affinity for the tissues in itself, even after "ripening" when it is largely converted into hematein. Some form of mordanting is ordinarily required; and most of the hematoxylin formulae either call for some metallic salt or specify previous treatment of the sections with one. In plant histology, however, there is some use for hematoxylin alone. Its greater affinity for plant than for animal tissue implies the presence of aluminium, copper, or iron in the former. In fact hematoxylin can be used as a very delicate reagent for iron or copper.

Perhaps the best known formulae for staining with hematoxylin are the combinations with aluminium, generally in the form of alum. Böhmer's alum hematoxylin (1865), altho no longer used, is of much historic interest as it was the first stain of this type to be used. The best known at present is Delafield's alum hematoxylin, which is very useful tissue stain with great affinity for chromatin and nuclei, and has much value in staining cellulose walls in vascular plants. Another alum hematoxylin used for similar purposes is that of Ehrlich.

Mayer's hemalum is another well known alum combination. In this stain hematein is first prepared and then combined with alum. The name hemalum, proposed by Mayer, is now generally accepted for this combination, and various other hemalum formulae have since been proposed. They are useful chromatin stains and are called for in various special procedures.

Mayer has also combined hematein with aluminium chloride, his hemacalcium calling for this salt and calcium chloride, while his muci-hematin contains aluminium chloride and glycerin. The latter is used for staining mucin.

The iron combinations are perhaps equally valuable. The original iron hematoxylin was that of Benda; but the best known at present is M. Heidenhain's, which is one of the most useful histological and cytological stains, both in botany and zoology. It is a powerful stain for chromosomes and centrosomes, and is of use for bringing out the middle lamellae in wood. Various other

modifications of iron hematoxylin have been used, but they are all similar in principle. Ordinarily the iron salt is not mixed with the stain, but is used for a preliminary mordanting of the tissue.

Hematoxylin has been combined with chromium, one of the early staining methods being that of R. Heidenhain, which called for potassium bichromate as a mordant. Various recent modifications are in use today, such as that of Apathy, for staining general tissue. Weigert uses a chrom combination for staining nervous tissue.

Benda uses hematoxylin following treatment with a copper salt for studying spermatogenesis; and Bensley a similar technic for chromosomes and mitochondria.

Mallory has proposed a formula for hematoxylin containing phosphomolybdic acid and also one containing phosphotungstic acid. The latter method is especially valuable for staining cells in the process of mitosis, and for distinguishing fibroglia, myoglia and neuroglia fibrils from collagen and elastin fibrils, especially in tumors, but also in normal tissues. It brings out sharply the striations in skeletal and cardiac muscle fibers. Hematoxylin is used in combination with other stains, especially eosin, but not so frequently as in the case of the common anilin dyes. The Van Gieson technic calls for hematoxylin followed by picric acid and acid fuchsin. A few other methods call for picric acid or ammonium picrate after hematoxylin: and it is sometimes used with eosin or after orange G or acid fuchsin. Most of these combinations, however, are called for only in the case of special procedures.

Delafield's hematoxylin*

Solution A		*Solution B*	
Ammonia alum, sat. aqu. sol.	400 cc.	Hematoxylin	4 g.
		Ethyl alcohol (95%?)	25 cc.

Mix the above solutions and allow to stand 3 to 4 days exposed to air and light; then filter. Then add to it:

Solution C

Glycerin	100 cc.
Methyl alcohol	100 cc.

Allow the mixture to stand until it becomes dark colored; it is subsequently kept in a tightly stoppered bottle, diluting considerably when used.

Ehrlich's glycerin alum hematoxylin:

Water	100 cc.
Absolute alcohol†	100 cc.
Glycerin	100 cc.
Glacial acetic acid	10 cc.
Hematoxylin	2 g.

*First published by Prudden 1885.

†Absolute alcohol is called for by the author; but on account of the large amount of water added there is no theoretical reason for not using 95% ethyl alcohol. Hematoxylin is readily soluble in alcohol of that strength.

The hematoxylin is dissolved in the alcohol, the acid added to it, then the glycerin and water. Alum is added in excess. The solution is ripened in an open flask in the light until dark red.

Heidenhain's iron hematoxylin:

1. Place sections for ½ to 3 hours, or longer, in a 1.5 to 4% solution of iron alum, $(NH_4)_2 Fe_2 (SO_4)_4$ (violet crystals).
2. Wash in water.
3. Place for half an hour in 0.5% solution of hematoxylin.
4. Wash in water.
5. Differentiate with the above iron alum solution, controling differentiation by microscopic examination.
6. Wash 5–10 minutes in running water.
7. Counterstain, if desired, with an orange or red acid dye.
8. Dehydrate and mount as usual.

Weigert's iron hematoxylin:*

Solution A		*Solution B*	
Hematoxylin	1 g.	Liquor feri sesquichlorati	4 cc.
Ethyl alcohol, 96%	100 cc.	Water	95 cc.
		HCl	1 cc.

For use mix equal parts of A and B. The mixture will keep several days, but is best prepared fresh.

Stain sections several minutes or longer. Wash in water. If desired, counterstain in the Van Gieson mixture of picric acid and acid fuchsin (See p. 40).

Mallory's phosphotungstic acid hematoxylin:

Ammoniacal hematein	0.1 g.
Water	100 cc.
Phosphotungstic acid (crystals)	2 g.

Dissolve the hematein in a little water by the aid of heat and add it when cool to the rest of the solution. If solution stains weakly at first it may be ripened by the addition of 5 cc. of a 0.25% aqueous solution of potassium permanganate, or it may be allowed to stand for a few weeks until it ripens spontaneously.

Hematoxylin may be used instead of ammoniacal hematein, but requires 10 cc. of the permanganate solution to ripen it.

Mayer's hemalum:

Hematein (or ammoniacal hematein)	1 g.
Ethyl alcohol (90%)	50 cc.
Alum	50 g.
Water	1 litre

After staining it is best to wash first with 1% aqueous alum solution, then with spring water. Dilution of the hemalum is best accomplished with 1% alum solution.

*From Mallory and Wright, 1924, p. 95.

CHAPTER X

THE THEORY OF STAINING

THRUOUT the preceding pages of this book an effort has been made so far as possible to avoid theoretical discussions. Altho they contain some statements the truth of which cannot be regarded as fully established, as in the case of the chemical composition of some of the dyes, the discussion in general has been confined to observations and to chemical information for which there is good authority, without any attempt to introduce explanations of a theoretical nature. The present chapter, dealing wholly with theory, is added because it is felt that a brief statement of some of the most probable theories to explain staining may be of value in assisting the histologist in the intelligent use of stains for his purposes.

A long theoretical discussion of this subject might be included here, basing it upon the lengthy arguments supporting the various theories that have appeared in the literature. Such a detailed discussion, however, would probably be of little value. Hence this chapter is confined to a bare outline of the important points of the different theories.

Theoretically the dyeing of textile fabrics and the staining of microscopic structures are the same. In one case only the gross effects are observed, in the other the microscopic details. Any theory, therefore, that will explain the details of microscopic staining will be fully adequate to account for dyeing in bulk.

Theories to account for dyeing or staining have in general been based exclusively upon either physical or chemical phenomena. It would seem at first thought that the dyes combine so firmly with the tissues stained by them that the phenomenon must be a chemical one; but the exponents of physical theories have taken pains to show that all the observed facts can be explained on a physical basis, and that some observations are hard to explain if a chemical union between tissue and dye actually takes place. In a chemical union a new substance is formed which does not necessarily have the properties of either substance entering into its formation, and it is ordinarily impossible to recover the original substances by means of simple solvents. When tissue is stained there is no evidence of any new substance having been formed, the colored tissue merely taking on one of the characteristics of the dye (color) in addition to the properties which it originally possessed; it is, moreover, ordinarily possible to extract all or nearly all of the color by sufficiently long immersion in water, or by the fairly brief action of alcohol. Another observation which points against chemical action is that the tissue never removes the dye completely from solution, even tho very dilute; whereas ordinary

chemical reactions tend to continue until one of the components of the reaction is exhausted. Such facts as these, to the exponents of the physical theory, are enough to refute the possibility of chemical action.

The Physical Theory. It has, indeed, been pointed out that all ordinary dyeing or staining phenomena can be explained on a physical basis. There are three factors, all purely physical in nature, which together or separately can explain many, if not all, staining phenomena. In the first place, as nearly all substances stained are more or less porous, simple physical forces such as capillarity and osmosis can account for penetration of the dye into the interior of the tissue. In the second place, the action of *ad*sorption can account for many staining phenomena, even for much of the selective staining with which the biologist is familiar. In the third place, a dye may penetrate some cellular element by *ab*sorption, remaining there in a state of solid solution. Any one or all of these three forces may operate in any given instance; and even the exponents of the physical theory of staining differ in the amount of weight given to each of them. The penetration of the dyes into the tissue by osmotic action is generally admitted; but some students of the subject favor adsorption, others absorption, as the primary explanation of staining phenomena.

The absorption or solution theory is very simple and the action of some dyes on some kinds of cellular tissue in the presence of mineral salts suggests that this factor is important, at least in some instances. It is also supported by the fact that a dye causes the tissue to become the same color as the dye shows in solution, but not necessarily the same as it shows in its dry form. Dry fuchsin, for example, is green; its solution, however, is red, and so are tissues stained by it, no matter how completely they may be dried. It is nevertheless difficult to explain all staining phenomena, particularly differential staining, on the basis of solution.

Adsorption, on the other hand, furnishes a very satisfactory explanation even of differential staining, and there are many who claim that all staining phenomena may be accounted for in this way. Adsorption is the property possessed by a solid body of attracting to itself minute particles of matter from a surrounding fluid; these particles may be compounds suspended in the fluid, or they may be ions incapable of existing independently except in solution. The principle of selective adsorption is well known to physical chemists, whereby certain ions may be adsorbed by certain substances much more readily than by others. Equally well known is the fact that the rate of adsorption of any ion is strongly influenced by the presence of other ions in the solution, and that an especially profound influence is exerted by the reaction of the solution, in other words by the concentration of free hydrogen or hydroxyl ions in the fluid; the influence of the latter

...about the adsorption bodies not being ...ence opposite from its effect upon the adsorption of acid dyes. Such principles as these, well established in physical chemistry, ... explanation of such phenomena as the ... staining of different cellular elements, ... of mordants, variations in rate of staining with changes in the salt content of ... solution, and influence of H-ion concentration upon the color assumed by tissue when exposed to the action of both acid and basic dyes.

Some of those who hold in general to the physical theory of staining admit that these simple physical phenomena alone cannot explain everything, as for example, instances in which a dye penetrates different cell elements equally readily, but can be easily extracted from some of them while scarcely at all from others. It is assumed, therefore, that the dyes penetrate the cells by mere absorption and diffusion, but are in some cases precipitated there by acids or bases, or other chemical reagents present, thus preventing their extraction by simple solvents. Such a theory admits the possibility of chemical action without assuming an actual chemical union between the dye and the tissue.

This precipitation theory would furnish a satisfactory explanation of the action of mordants. It is well known that certain tissues, that stain feebly if at all with certain dyes, "take" these same dyes deeply if previously treated with the proper chemical. Such a mordant, already present in the tissue, might well cause the precipitation of the dye inside the cell walls. On the other hand the action of mordants may be equally readily explained if it is assumed that staining takes place by means of adsorption or as a process of solution. In the first place, all mordants contain ions that are known to have a decided influence upon the rate of adsorption, and their action may be thus accounted for; they also are nearly always substances that decrease the solubility of any dye and thus increase the readiness with which a dye may enter into solution within the tissue to be stained, if one accepts the solution theory. In other words, the action of mordants can be accounted for by any of the theories of staining.

Evidence is still lacking, in fact, to prove or to disprove any of these physical theories on the one hand or the chemical theory on the other. The difference, perhaps, is not one of fundamental importance. All of the theories teach about the same practical ... to the type of dye to select for any particular ... to the influence of salt content or H-ion concentration ... solution of tissues upon the rate of subsequent staining, ... moreover, frequently pointed out that there is no sharp distinction between chemistry and physics, and in each process ... involved in staining we may ... the ... between the two branches of science. Where ...

[illegible] that a given phenomenon is purely physical or purely chemical. There are, however, certain chemical principles [illegible] from the physical ones just mentioned, that [illegible] the phenomenon of staining; and it is these [illegible] most important by the exponents of the chemical theory.

The Chemical Theory. It is claimed on behalf of the chemical theory that just because physical forces alone can explain the facts one is not justified in assuming that chemical unions do not take place when the opportunity for them is present. It is agreed that some parts of the cell are acid in reaction, others alkaline; and it is a well known chemical principle that the former would tend to combine with the kations in solutions with which they come in contact, the latter with the anions. Now inasmuch as in certain dyes the color exists in the kation (basic dyes) and in others in the anion (acid dyes), it is natural to expect chemical combinations to take place between dye and tissue, depending upon the reaction of the latter. Arguments for the physical theory which exclude chemical action must furnish strong proof that no chemical union occurs; and those who favor the chemical theory claim that such proof is lacking. That the stained tissue does not present any characteristics to the eye not possessed by either tissue or dye before staining does not prove that no new substance has been formed, nor is this claim refuted by the fact that sufficiently long action of solvents removes the color. Alcohol and even water are not absolutely inert chemically and may withdraw the dye by chemical instead of physical action; the very length of time necessary to remove the color completely (sometimes so long as to allow bacterial decomposition of the tissue) indicates that a rather strong union between dye and tissue has taken place. As to the fact that a tissue which has a strong affinity for some particular dye never withdraws that dye completely from a very dilute solution, those who favor the chemical theory point out instances where chemical reactions are known to take place and yet to stop before either component is exhausted; and they further claim that chemical action is strongly indicated by the fact that in dilute solutions the tissues take up relatively larger quantities of the dye than in concentrated solutions.

In brief, the chemical theory of staining is that the tissues have certain definite chemical affinities which are satisfied by the chemical affinities of the dyes; therefore, when the tissue is put in a solution of the dye the latter combines with those portions of the tissue or of the individual cells which have the proper chemical nature. This theory, it will be seen, is especially well adapted to explain the differential staining which takes place when we find [illegible] staining only the nuclei. It must be remembered, however, that it is merely a theory. As a matter of fact, the

probabilities are that staining is both a chemical and a physical phenomenon.

The chemical theory of staining is dependent largely upon the question of the acid or the basic character of the dye molecule. It will be recalled that all ordinary dyes are encountered either as sodium or potassium salts of dye acids or as dye salts of colorless acids, the former being the acid dyes and the latter the basic dyes; while certain compound stains are neither acid nor basic dyes, inasmuch as the property of color exists in both the anion and the kation.

The fundamental principle involved in this theory is that certain parts of animal or plant cells are acid in character and hence have an affinity for the basic dyes. The nuclei of the cells, or especially the chromatin within the nuclei, are assumed to be acid in character (due largely to their constituent nucleic acid), and there is no question but that they have a strong affinity for basic dyes; while the cytoplasm has an affinity for acid dyes and is assumed to be basic in character.

This theory assumes that the acids and bases which go to make up body tissue are ordinarily amphoteric, capable of acting as bases in acid solutions and as acids in basic solutions, the H-ion concentration at which any such compound changes from an acid to a base in its action being known as its isoelectric point. It also assumes that these compounds, altho insoluble, act as tho they were electrolytes dissolved in any fluid in which they are immersed. Hence, on this assumption, such a compound acts as a base or as an acid in any staining solution according to whether its isoelectric point is below or above the H-ion concentration of that solution. The chemical theory therefore postulates that any cellular element takes a basic dye if the H-ion concentration of the staining solution is below its isoelectric point, an acid dye if it is above that point.

Now, exactly such a phenomenon as this is to be observed in staining any tissue. With very acid staining solutions even the nuclei take the acid dyes; if one employs successively a series of solutions of decreasing H-ion concentration, the affinity of the nuclei for the acid dyes rapidly becomes less; till at a fairly definite reaction, usually in the region of pH 4, they lose their affinity for acid dyes and take the basic dyes. In solutions near neutrality, therefore, the nuclei take basic dyes, the cytoplasm the acid dyes. Finally at a point considerably to the alkaline side of neutrality even the cytoplasm takes the basic dyes. Such an observation is interpreted to mean that the isoelectric point of the nuclei lies considerably to the acid side of neutrality, that of the cytoplasm considerably to the alkaline side. (This is merely another way of making the statement, given two paragraphs above, that the nuclei are acid in character, the cytoplasm basic.) Now assuming

that this interpretation is correct, it is possible to determine the isoelectric points of different parts of the cell by staining at different reactions. Efforts have recently been made, in this way, to determine the isoelectric points of different species of bacteria, and it has been claimed that the difference between Gram-positive and Gram-negative organisms is really a difference in isoelectric point, while the Gram-variable bacteria have isoelectric points so near the reaction of the ordinary staining solutions that they fail to give a sharp Gram-reaction one way or the other.

Now this is by no means the whole of the chemical theory of staining. It is difficult to explain wholly on the basis of various isoelectric points the fact that certain basic dyes have stronger affinities for certain parts of the nuclei than for others, and that of the various cytoplasmic structures outside the nucleus some are more readily stained by certain acid dyes and some by others. In the Flemming triple stain for example, which employs the acid dye orange G and the two basic dyes safranin and gentian violet, with intervening alcoholic differentiation, it is possible to stain the chromatin with gentian violet and the rest of the nucleus with safranin. It is difficult to say just how any chemical theory of staining can yet satisfactorily explain such selective action as this. It is, indeed, admitted by some upholders of the chemical theory that the chemical action of dyes is not specific, and merely serves to differentiate acid from basic elements of the tissue; and that the further differentiation, as between chromatin and other parts of the nucleus, is due to physical forces.

Discussion of the Chemical Theory. The weaknesses of the chemical theory show up particularly in reactions such as this triple stain. Besides the difficulty of accounting for the stronger affinity of certain portions of the nucleus for certain basic dyes and other portions of the nucleus for other basic dyes, there is the difficulty of explaining the action of solvents. The differential staining secured in the Flemming stain, and in fact in the majority of other similar procedures, is brought about not by the staining itself but by the action of solvents which extract some of the dyes more readily from certain portions of the cell than from others. In case a chemical union has taken place between the tissue and the dye, the alcohol or other solvent used must have the effect of breaking down the chemical compound formed between the tissue proteins and the dye molecule, or else it must actually dissolve out the compound in question. It is, however, difficult on the one hand to conceive of alcohol breaking down such a compound; while, on the other hand, if the compound formed is dissolved out of the tissue, it is hard to understand how restaining of decoloured structures is possible.

A further difficulty of the chemical theory arises from the fact that it assumes ionization of the compounds stained, while the

[illegible]

Dye can, however, assume that differential staining [illegible] differences in chemical nature of the different parts of the cell without adopting the generally accepted chemical theory. Even if the dye is actually taken up by a process of adsorption, this process will be greatly influenced by the chemical nature of the different parts of the cell. Substances of an acid character would adsorb basic dyes more readily, and retain them in the adsorbed state more firmly, than they would acid dyes, and substances of a basic character would adsorb acid more readily than basic dyes. Amphoteric substances are usually acid or basic in their prevailing character. Acid substances such as cell nuclei exert a decided chemical affinity for basic dyes and accordingly adsorb them readily. They have, however, inferior degrees of affinity for acid dyes and in order to obtain staining with such dyes it is only necessary to convert them into a favorable physical form for adsorption. Basic substances, on the other hand, such as cytoplasm, altho ordinarily stained only by acid dyes, may be stained by basic dyes if the latter are employed in a favorable physical form. Favorable conditions for these unusual types of staining are obtained merely by altering the reaction of the staining solutions. Thus one does not have to assume chemical combination between dye and tissue in order to account for the different type of staining obtained in solutions of varying pH value.

It is extremely easy to theorize in regard to the mechanism of staining but very difficult to propose any conclusive argument. Dye chemists have obtained actual quantitative data as to withdrawal of dyes from solution by the fibres of which textiles are made up, which points very strongly to the process being physical rather than chemical. These conclusions are not directly applicable to biological staining for two reasons; in the first place fixed tissues are decidedly different from the animal or vegetable fibres studied by the dye chemists and quantitative data seem to be lacking as to how such tissue removes any dye from solution. In the second place, staining effects are in most instances brought about by the action of decolorizing agents after the staining has taken place, and thus introduces a factor which dye chemists have not had to take into account in explaining the affinity of textiles for dyes.

Theories of Staining. It is probable, therefore, that whether the original combination between the dyes and the tissue is physical or chemical, differential staining indicates some chemical difference between cell elements thus differentiated. In this way the use of stains perhaps forms a connecting link between the two branches, histology and microchemistry. These two branches of science are generally thought to be entirely distinct. The histologist, with the technic and viewpoint of the biologist, prepares selected sections of various materials colored with one or more of a long series of available dyes, and studies the biological structures present under the microscope. The microchemist, with the technic and viewpoint of the chemist, examines with the microscope similar material treated with various reagents of known chemical reaction, and from his observations draws conclusions as to the chemical nature of the substances examined. There is some possibility, however, that the difference between histology and microchemistry is one of point of view rather than of methods. Both the microchemist and the histologist study the action of chemical compounds on substances or structures visible under the microscope; the difference is that the microchemist uses the chemical compounds in question as chemical reagents while the histologist uses his as dyes to color the microscopic structures and thus to increase their visibility.

Now, on any theory of staining, it is apparent that the stains are reagents for bringing out differences of a physical or chemical nature between the different proportions of the tissue. The exact action of these reagents, however, is unfortunately not yet understood. To bridge the gap between histology and microchemistry these reactions must be made intelligible. A step in this direction was recently taken by Unna (1921) in a very important contribution to the subject of cell chemistry. He pointed out the need of harmonizing chemical and histological investigations and proposed a method of doing this which he called *chromolysis*. The technic he developed, altho quite complicated in its details can be summed up briefly as follows: to select a dye, or a mixture of dyes, either acid or basic, which bring out some intracellular structure whose chemistry it is desired to learn; then to submit the sections to the action of various solvents, beginning with simple cold water, next proceeding to hot water, and from that to the more powerful solvents, but using only those whose action on proteins, lipoids or carbohydrates is known to the chemist; then to stain the sections with the staining fluid selected; and finally to determine by microscopic examination which solvents have removed the substance under investigation.

By such methods as these progress in the microchemistry of the cell may be possible, and it will be readily seen that once the gap between chemistry and histology is bridged, progress will become

constantly more and more rapid. As soon as it is possible to obtain a reasonable hypothesis as to the chemical nature of the various cellular elements in any particular type of tissue, then some conclusions can be drawn as to the affinities of the various stains for the chemical compounds thus recognized. Subsequently the use of the same stains on other tissue should apply the information thus obtained to the solution of the chemistry of other microscopic structures. In other words, stains will then become chemical reagents instead of merely dyes for making microscopic structures visible. In this way it is hoped that chemistry and histology, working together, may solve some of the obscure problems as to the nature of the cell and its contents.

APPENDIX I

TABLES RELATING TO STAINS

In the following tables all the dyes that are frequently mentioned in the literature dealing with microscopic technic are listed, together with the most important uses of each in the biological laboratory. The list of uses (Table 2) is necessarily incomplete. In the case of the most commonly used stains, in particular, it has been necessary to group various uses together under some general term, without attempt to list the individual procedures. In general, however, the policy has been to list the methods for which a stain is most commonly used today, and of the obsolete methods to give only those of historical interest. Criticism will be welcomed from anyone noticing any serious omission.

The dyes in Tables 1 and 2 are listed in the same order as in the main part of this book. Hence either the general Table of Contents or the list in Table 1 may be used to learn the order of the stains when it is desired to find some particular one in Table 2. For an alphabetical list referring to the following pages see the general Index.

The references given in the last column of Table 2 refer ordinarily by name and date to the literature listed in Appendix III. Certain references, however, are used so often that the date is omitted: they are referred to merely as *Chamberlain*, *Lee*, *Ehrl. I*, or *II*, *Krause*, and *Mal. & Wr.* These refer respectively to: Chamberlain's Methods in Plant Histology (1924), Lee's Microtomists Vade-mecum (1921), Ehrlich's Enzykopädie der Mikroskopischen Technic (1910), Vol. I and Vol. II, Krause's revision of the same (1926–7), and Mallory and Wright's Pathological Technic (1924). No effort has been made to give the original reference in all cases; but rather to refer to some readily available description of each technic that can be followed by anyone using the procedure.

TABLE 1. NOMENCLATURE AND SYNONOMY OF STAINS.

Colour Index No.	Schultz* No.	Preferred Designation	Synonyms and slightly varying shades	Page reference
		(a) Synthetic Dyes		
7	5	Picric acid		
8	6	Martius yellow	Naphthol yellow. Manchester yellow.	
12	IV 2	Aurantia	Imperial yellow	
13	157	Fast yellow	Acid yellow	
20	25	Chrysoidin Y	Brown salt R. Dark brown salt R.	
27	36	Orange G†	Patent orange. Acid orange G, GG, GGO. Crystal orange GG. Wool orange 2G, (Slightly differing grader Orange GG, GMP)	
75	76	Oil red O	Sudan II. Oil scarlet. Fast oil orange II. Red B. Fat ponceau. Orange RR.	
88	112	Bordeaux red	Fast red B or P. Cerasin. Archelline 2B. Azo-bordeaux. Acid bordeaux.	
133	194	Janus green B	Diazin green S. Union green B.	
142	138	Methyl orange	Orange III. Helianthin. Gold orange. Tropaeolin D	
143	139	Orange IV	Orange N. Acid gold D. Tropaeolin OO	
150	144	Orange I	Naphthol orange. Tropaeolin G, OOO No. 1	
151	145	Orange II	Gold Orange. Orange A, P, R. Acid orange. Orange extra. Mandarin G. Tropaeolin OOO No. 2	
180	IV 104	Narcein		
184	168	Amaranth	Naphthol red. Fast red. Bordeaux. Bordeaux SF. Victoria rubin. Azo rubin. Wool red.	
212		Methyl red		
258	223	Sudan III	Sudan red. Tony red. Scarlet G or B. Fettponceau G. Oil red. Cerasin red.	
259	252	Sudan IV	Scarlet red. Fettponceau. Ponceau 3R.	
280	247	Biebrich scarlet, water soluble	Croceine scarlet. Ponceau B.	
281	253	Bismarck brown Y	Vesuvin. Phenylene brown. Manchester brown. Zanzibar brown. Leather brown. (Slightly differing grades Bismarck brown G)	
370	367	Congo red	Congo. Cotton red. Diphet red.	
438	359	Trypan red		

446	363	Benzopurpurin 4B	Cotton red 4B. Dianil red 4C. Diamine red 4B. Sultan 4B. Direct red 4B.	58
456	370	Vital red	Brilliant Congo R. Brilliant Congo red R. Acid Congo R. Azidine scarlet R. Brilliant dianil red R.	59
465	379	Dianil blue 2R	Direct steel blue BB. Benzo new blue 2B. Naphthamine brilliant blue 2R.	54
477	391	Trypan blue	Diamin blue 3B. Benzo blue 3B. Dianil blue 3BX. Congo blue 3B. Naphthamine blue 3BX. Benzamin blue 3B. Azidin blue 3B. Niagara blue 3B.	54
1037	778	Alizarin	(Various grades denoted as: Alizarin P, VI, Ie).	55
1034	780	Alizarin red S	Alizarin monosulfonate of sodium. Alizarin red WS. Alizarin carmin.	56
1037	783	Purpurin	Alizarin No. 6. Alazirin purpurin.	58
920	II 348	Thionin	Lauth's violet. (Not thionin blue, which is Schultz No. 661).	58
...	...	Azure C		61
...	...	Azure A	Methylene azure. Azure I.	62
...	...	Azure B		64
922	659	**Methylene blue, Med: U. S. P.**	Swiss blue. (Slightly different grades: Methylene blue BX, B, BG and BB).	64
...	...	Methylene violet, Bernthsen	Not methylene violet RRA or 3RA, Schultz No. 680).	70
924	660	Methylene green		70
925	IV 592	Toluidin blue O.	Methylene blue O.	71
927	663	New methylene blue N	Methylene blue NN.	72
877	621	Brilliant cresyl blue	Cresyl blue. Cresyl blue 2RN or 2BS. Brilliant blue C.	72
883	626	Gallocyanin	Alizarin blue RBN. Chrome blue GCB. Fast violet.	73
894	637	Gallamin blue		74
900	641	Celestin blue B	Coreine 2R.	74
908	648	Resorcin blue	Fluorescent blue. Iris blue. Often called lacmoid.	74
913	653	Nile blue sulfate	Nile blue A.	75
...	...	Cresyl violet	Cresyl echt violet.	75
825	670	Neutral red	Toluylene red.	78
826	669	Neutral violet		79
841	679	**Safranin O**	(Slightly different shades: Safranin AG, T, MP, Y and G.)	79

*The Schultz No. refers ordinarily to the sixth edition of Schultz's Farbstofftabellen (1923). When the number is preceded by a Roman numeral, the dye in question is not listed in the fifth edition and the Roman numeral indicates the edition in which it is to be found.

†The dyes printed in bold faced type are the most commonly used stains.

Colour Index No.	Schultz No.	Preferred Designation	Synonyms; and slightly varying shades	Page reference
682	686	Amethyst violet	Heliotrope B. Iris violet.	83
683	694	Magdala red	Naphthalene red, Naphthalene pink. Naphthylamine red. Sudan red.	85
864	700	Nigrosin, water soluble	Nigrosin W, WL, etc. Gray R, B, BB. Silver gray. Steel gray. Indulin black.	84
655	493	Auramin	Pyoktaninum aureum. Pyoktanin yellow. Canary yellow.	88
657	495	Malachite green	Emerald green. New Victoria green. Diamond green. Solid green. Light green N.	89
662	499	Brilliant green	Ethyl green. Malachite green G.	89
670	505	Light green SF, yellowish	Light green 2G, 3G, 4G, 2GN. Acid green (with various shade designations). Fast green N.	90
...	...	Fast green FCF		91
676	511	Pararosanilin	Basic fuchsin.* Basic rubin. Para-fuchsin. Para-magenta. Anilin red.	92
...	...	Rosanilin	Basic fuchsin. Magenta. Rubin.	92
678	518	New fuchsin	Isorubin. Fuchsin NB.	92
692	524	Fuchsin, acid	Fuchsin S, SN, SS, ST, or S III. Acid magenta. Acid rubin.	97
682	518	Ethyl violet	Ethyl purple 6B	100
679	514	Hoffman violet	Dahlia. Iodine violet. Primula R. Red violet. Violet R, RR, 4RN.	100
680	515	Methyl violet	Dahlia B. Paris violet. Gentian violet. Pyoktanin blue. (Various shades denoted: Methyl violet 8R, 2R, R, B, 2B, 3B, BBN, BO, and V3).	101
...	...	Gentian violet†	Violet C, G or 7B. Gentian violet. Hexamethyl violet. Methyl violet 10B.	103
681	516	Crystal violet		104
684	519	Methyl green	Double green. Light green.	109
656	II 284	Iodine green	Hoffman's green	110
689	681	Spirit blue	Anilin blue, alcohol soluble. Gentian blue. Night blue. Lyons blue. Paris blue.	111
706	688	Methyl blue	Cotton blue. Helvetia blue.	112
707	539	Anilin blue, water soluble	China blue. Soluble blue 3M or 2R. Marine blue. Cotton blue. Water blue. Berlin blue.	113

724	555	Rosolic acid	Aurin	115
724	555	Corallin, yellow	Sodium salt of rosolic acid.	116
726	556	Coraflin red	Aurin R	116
739	568	Pyronin G		118
741	...	Pyronin B		118
749	573	Rhodamine B	Rhodamine O. Brilliant pink.	119
766	585	Fluorescein	Uranin	123
768	587	Eosin, yellowish	Eosin. Water soluble rosin. Eosin Y, W or WS. (Various grades denoted as Eosin G, Y extra, S extra, J extra, B extra, GGF, 3J, 4J, KS, DH, and JJF.)	123
769	588	Methyl eosin	Eosin, alcohol soluble.	124
770	589	Ethyl eosin	Eosin, alcohol soluble. Eosin S.	125
771	590	Eosin, bluish	Eosin BN, B, BW, DHV. Safrosin. Eosin scarlet B, BB. Scarlet J, JJ, V. Nopalin G. Caesar red.	127
...	...	Mercurochrome 220		127
772	591	Erythrosin, yellowish	Erythrosin R or G. Pyrosin J. Iodo-eosin G.	128
773	592	Erythrosin, bluish	Erythrosin B. Pyrosin B. Eosin J. Iodo-eosin. Dianthine B. (Slightly different shades: Erythrosin D, J, JNV, W).	128
774	593	Phloxine	Erythrosin BB, or B extra. New pink.	130
778	596	Phloxine B	Phloxine TA, N, or BB. Cyanosine. Eosin 10B	130
779	597	Rose bengal	(Various grades denoted as: Rose bengal B, 2B, 3B).	132
790	...	Acriflavine		141
			(b) Natural Dyes	
1177	874	Indigo	Indigo blue	149
1180	877	Indigo carmin	Indigotine Ia.	150
1239	932	**Cochineal**	(The aqueous extract of the cochineal insect).	150
		Carmin	(The lake prepared by adding alum to cochineal).	151
		Carminic acid	(The active dye purified from cochineal).	151
1242	934	Orcein		153
1243	935	Brazilin		154
1246	938	**Hematoxylin**	(The ether extract of logwood).	155
		Hematein	(The dye formed on oxidation of hematoxylin).	156

*At least three different dyes are apparently sold to biologists as basic fuchsin. It is not yet known which is the best or whether the different products may not be suited to different biological purposes.

†Nearly synonymous with methyl violet; various mixtures of methyl and crystal violets are sold under this name.

TABLE 2. THE PRINCIPLE USES OF THE MOST IMPORTANT STAINS.

Name of stain and page reference*	Name or author of technic	Application	Bibliographic references†
Picric acid p. 40		For cytoplasm. Contrast stain.	Lee, p. 176. Krause, p. 1900.
	Van Gieson	With acid fuchsin; for connective tissue. See p. 40‡	Lee, p. 176. Krause, p. 407, 9059.
Martius Yellow p. 41	Pianese	For cancer tissue, with acid fuchsin and malachite green.	Pianese, 1896.
		Same technic adapted for staining pathologic plant tissue (fungus diseases). See p. 41	Müller, 1914. Weigert, 1884.
Aurantia p. 42		For light filters in photomicrography.	Krause, p. 1384.
	Champy-Kull	For mitochondria; with acid fuchsin and toluidin blue.	Lee, p. 381.
Fast Yellow p. 43	Shaffer.	For bone sections	Shaffer, 1898. Ehrl. I, p. 978.
	Unna.	Combined with eosin, phloxine and anilin blue; in the study of chromolysis.	Unna, 1897.
Chrysoidin Y p. 43		A general cytoplasm stain; a good substitute for Bismarck brown.	
		A valuable plasma stain in sections of tissue. See p. 44	Ehrlich, 1886. Lee, p. 176.
		Background stain for hematoxylin and other nuclear stains in botanical histology.	Chamberlain, p. 98.
	Flemming triple stain	As cytoplasm stain, in contrast to crystal violet and safranin. See p. 31	Flemming, 1891. Lee, p. 176. Ehrl. I, p. 975–7. Krause, p. 1790.

Orange G p. 44	Mallory's connective tissue stain	For cytoplasm and red blood cells; with anilin blue and acid fuchsin. See p. 114	Mallory, 1900. Mall. & Wr., p. 118. Kyrle, p. 88.
	Ehrlich-Biondi-Heidenhain.	For tissues; with methyl green and acid fuchsin.	Kyrle, p. 437. Lee, p. 178, 9.
	Ehrlich triacid mixture.	For blood; combined with methyl green and acid fuchsin.	Ehrl. II, p. 215. Krause, p. 457; 1707.
	Bensley's neutral gentian.	Combined with gentian violet; for islands of Langerhans.	Bensley, 1911.
Oil Red O p. 45	French.	For staining fat; as substitute for Sudan III or IV.	French, 1922.
	Proescher.	With pyridin; for staining fat. See p. 45	Proescher, 1927.
Bordeaux red p. 45		For cytoplasm, before staining with Heidenhain's hematoxylin.	Krause, p. 243.
	Gråberg.	For sections of spleen, testis and liver, with methyl green and thionin.	Krause, p. 243.
Janus green B p. 46		For vital staining of chondriosomes. See p. 46	Michaelis, 1900. Bensley, 1911. Lee, p. 232–3.
		For sections of embryos; with neutral red.	Faris, 1924.
Methyl orange p. 47	Bergonzini.	In place of orange G in the Ehrlich-Biondi stain.	Bergonzini, 1891. Krause, p. 909.
	Ebbinghaus.	For keratin in sections of skin.	Ebbinghaus, 1903. Krause, p. 909.
		For determining reaction of cell sap in plants.	

*The pages listed in this column refer to the present book.
†The references in this column are to the list in appendix III, p. 204.
‡The pages named in this column in bold faced type refer to the places in this book where the procedures in question are given in detail.

Name of stain and page reference*	Name or author of technic	Application	Bibliographic references†
Orange II p. 48	French	As cytoplasm stain; with eosin and azure C. See p. 62‡	French, 1926.
Narcein p. 48	Ehrlich.	As component of "neutral" stain mixtures.	Ehrlich and Lazarus, 1898, p. 25–7.
Amaranth p. 48	Griesbach.	For axis cylinders.	Griesbach, 1886.
Sudan III p. 49	Daddi.	For fat in tissues.	Daddi, 1898. Lee, p. 358–9. Krause, p. 723–31.
	Bugnon	For differentiating suberized and cutinized tissue in plants.	Bugnon, 1919.
Sudan IV p. 50	Michaelis. Herxheimer.	For fat in tissues. See p. 50	Michaelis, 1901. Lee, p. 358–9. Krause, p. 723–31.
Biebrich Scarlet, W. S. p. 51		A general plasma stain.	Krause, p. 169.
	Pelagetti.	For cytoplasm, after polychrome methylene blue or Unna's hematein.	Pelagetti, 1904.
	Paladino.	Mixed with alum hematoxylin; for staining tissue sections.	Paladino, 1895.
	Bowie.	In neutral stain combination with ethyl violet, for staining islets of Langerhans.	Bowie, 1924.
Bismarck Brown Y p. 51		A general plasma stain, formerly much used. A good mucin stain. Good for vital staining and for staining in bulk.	Krause, p. 173, 716, 2070.
		For bacteria, particularly in contrast to gentian violet in the Gram technic.	Hucker and Conn, 1923. Mal. & Wr., p. 273.

	Foot and Strobell.	Chromosomes in smear preparations of eggs.	Foot and Strobell, 1905.
Congo red p. 52		Background stain for hematoxylin and other nuclear stains.	Carnoy and Lebrun, 1897. Lee, p. 178, 408, 453. Krause, p. 413.
	Griesbach.	For axis cylinders.	Griesbach, 1886.
	Schaffer.	For sections of embryos.	Schaffer, 1888.
		For staining plant mucin.	
	Klebs.	Reagent for cellulose.	Klebs, 1886.
	Blackman	As stain for Uredineae	Blackman, 1905.
	Matsuura.	For staining elastic tissue.	Matsuura, 1925.
Trypan red p. 52		For vital staining.	Lee, p. 388–90.
Benzopurpurin, 4B p. 53	Zschokke	Plasma stain, especially in contrast to hematoxylin.	Lee, p. 179. Krause, p. 160. Zschokke, 1888.
		For vital staining.	Lee, p. 390.
Vital red p. 53	Evans.	For vital staining	Dawson, Evans and Whipple, 1920.
Dianil blue 2R p. 54		For vital staining.	Corner and Hurni, 1918. Sutter, 1919.

*The pages listed in this column refer to the present book.
†The references in this column are to the list in appendix III, p. 204.
‡The pages named in this column in bold faced type refer to the places in this book where the procedures in question are given in detail.

Name of stain and page reference*	Name or author of technic	Application	Bibliographic references†
Trypan blue p. 54		For vital staining.	Lee, p. 388–90.
Alizarin red S p. 56	Benda	For chromatin; in combination with crystal violet (chromatin brown, mitochondria violet).	Lee, p. 322. Krause, p. 1562.
	Schrötter	For sections of nervous tissue.	Lee, p. 408.
		Vital stain for nerve tissues.	
Purpurin p. 56		A nuclear stain.	Krause, p. 1985.
		Reagent for detecting insoluble calcium salts in protoplasm.	Krause, p. 1986.
Thionin p. 58		For chromatin and mucin in general histology; and as general nuclear stain. See p. 59‡	Lee, p. 168. Krause, p. 2159.
		For frozen sections of fresh tissues. See p. 59	
	Ehrlich.	For amyloid (colored blue), mast cells and mucin (red).	Ehrl. II, p. 78. Krause, p. 1377.
	Gråberg.	With methyl green and bordeaux red, for sections of spleen, testis and liver.	Krause, p. 243.
	Frost.	Staining very young bacterial colonies on "little plates" (i. e., thin layers of bacteriological media on microscopic slides). See p. 60	Frost, 1916.
Azure C p. 61	French	As nuclear stain; with eosin and orange II. See p. 62	French, 1926b.
Azure A p. 62	Haynes.	As nuclear stain; preceding eosin or following phloxine. See p. 63	Haynes, 1926, a & b.
	Giemsa.	In neutral stain combination with eosin; for staining blood, protozoa, etc.	Krause, p. 189.

	MacNeal	Constituent of tetrachrome stain; for staining blood. **See p. 147**	MacNeal, 1922.
Methylene blue p. 64		A widely used nuclear stain in general histology (zoological), with many special applications.	Lee, p. 186–96. Krause, p. 1386–7.
	Unna. Goodpasture. Terry.	Various formulae of polychrome methylene blue; as tissue stain. **See p. 68**	Mal. & Wr., p. 76. Terry, 1928.
		A favorite bacterial stain, used for many special purposes, as in examination of milk and diagnosis of diphtheria. **See pp. 67–69**	Krause, p. 1387.
	Mallory	As nuclear stain, in contrast to phloxine, on pathological or other histological material. **See p. 131**	Mallory, 1904. Mal & Wr. p. 100–2. Lee, p. 181.
	Ehrlich.	Staining small animals intra vitam. Vital staining of nervous tissue.	Lee, p. 188–195. Krause, p. 718, 1390.
	Frothingham.	Mixed with basic fuchsin; for nervous tissue, in diagnosis of rabies. **See p. 97**	Mal. & Wr., p. 440
	Romanovski, and others.	For staining blood; in combination with eosin. **See pp. 144–148**	Krause, p. 188–9
	Levine.	With eosin, as indicator in bacteriological media, for differentiating colon and aerogenes organisms.	Levine, 1921.
Methylene green p. 70		For wood and fixed chromatin, in plant sections. (Does not stain fresh chromatin.)	
Toluidin blue O p. 71	Pappenheim's panchrome stain	A metachromatic nuclear stain often useful in place of thionin or methylene blue.	Krause, p. 2187.
	Albert	Diagnosis of diphtheria. **See p. 71**	Albert, 1920.

*The pages listed in this column refer to the present book.
†The references in this column are to the list in appendix III, p. 204.
‡The pages named in this column in bold faced type refer to the places in this book where the procedures in question are given in detail.

Name of stain and page reference*	Name or author of technic	Application	Bibliographic references†
Brilliant cresyl blue, p. 72		For blood; to bring out platelets and reticulated cells. See p. 73‡	Robertson, 1917.
	Irwin	As a vital stain.	Irwin 1927.
Gallocyanin p. 73		Nuclear stains; used in form of iron lakes.	Proescher and Arkusch, 1928.
Gallamin blue p. 74			
Celestin blue B p. 74			
Resorcin blue p. 74	Tsvett.	As microchemical reagent for detection of callose.	Tsvett, 1911.
Nile blue sulfate p. 75	Lorrain Smith.	For fats; to distinguish between fatty acids and neutral fat. See p. 75	Smith and Mair, 1911.
		Supravital stain for embryos.	Detwiler, 1917.
		Vital stain for hydrae.	Weimer, 1927.
Cresyl violet p. 75		For fixed tissue, (especially of tumors).	Lee, p. 184.
		For nervous tissue.	
		For vital staining of blood.	Spiridonovitch, 1924
		For fresh tumor tissue.	Williams, 1923.

Neutral red p. 78		For histological tissue (of embryos); contrast stain to janus green.	Faris, 1924.
		Nuclei, especially of blood cells, and Nissl granules of nerve cells, stained intra vitam.	Lee, p. 179. Krause, p. 1716–9
		"Vital staining" of blood, (i. e., fresh in moist chamber); also of fresh gonorrhoeal pus.	Lee, p. 382. Krause, p. 1717.
		As indicator in bacteriological culture media; for distinguishing colon from typhoid organisms, and other similar purposes.	Rothberger, 1898.
	Twort	With light green, as stain for parasites in tissue.	Twort, 1924.
Neutral violet p. 79	Unna.	Used in study of chromolysis.	Unna, 1921.
Safranin O p. 80		A widely used nuclear stain. **See pp. 81–83**	Lee, p. 165–7,352,391. Krause, p. 2041–5.
		For tissues of vascular plants, in combination with variety of contrast stains. Cutinized, suberized, and lignified tissue. Spore coats. Protein.	Chamberlain, p. 56, 88–90.
	Flemming's triple stain.	For chromatin and other nuclear elements, in conjunction with gentian violet; orange G as contrast stain. **See p. 81**	Flemming, 1881. Lee, p. 177. Ehrl. I, p. 475–7. Krause, p. 1736.
		For chromatin; with light green. **See p. 82**	Benda, 1891. Lee, p. 181.
		For bacteria, particularly in contrast to gentian violet in the Gram technic.	Hucker and Conn, 1923. Mal. & Wr., p. 273.
	Flemming.	For nuclei.	Flemming, 1881.

*The pages listed in this column refer to the present book.
†The references in this column are to the list in appendix III, p. 204.
‡The pages named in this column in bold faced type refer to the places in this book where the procedures in question are given in detail.

Name of stain and page reference*	Name or author of technic	Application	Bibliographic references†
Magdala red p. 88	Kultschitzky.	For elastic tissue.	Kultschitzky, 1895.
Nigrosin p. 84		For central nervous tissue; alone or with other stains.	Krause, p. 1724.
	Jarotzky.	For tissue of pancreas; following hematoxylin.	Krause, p. 1724.
		For algae and fungi.	Chamberlain, p. 63.
	Unna.	Combined with "orange" (orange G?); used in study of chromolysis.	Unna, 1921.
	Pfitzer's picro-nigrosin.	For chromatin.	Pfitzer, 1883. Lee, p. 182, 350, 408.
	Dorner	With basic fuchsin in staining bacterial spores. See p. 85‡	Dorner, 1926.
Auramin p. 88	Fischel.	Vital staining of salamander larvae.	Fischel, 1901. Krause, p. 135.
	Vinassa.	For plant sections.	Vinassa, 1891. Krause, p. 135
Malachite green p. 89	Pianese.	In combination with acid fuchsin and martius yellow; for cancer tissue.	Pianese, 1896 Mal. & Wr., p. 83.
		For host tissue in plants infected with fungi; used in the Pianese combination.	Müller, 1912. Vaughan, 1914.
	v. Beneden.	For Ascaris eggs.	Krause, p. 1353.
	Petroff.	For erythrocytes.	Id.
	Maas.	Contrast stain, following borax carmin.	Id.

Brilliant green p. 89	Krumwiede.	Indicator in bacteriological media for differentiating organisms of colon, typhoid and dysentery groups.	Mal. & Wr., p. 228–9.
		For inhibiting *B. coli* in stools.	Browning, Gilmore & Mackle, 1918.
		Enrichment of the typhoid organism in broth cultures.	Torrey, 1919.
Light green SF yellowish p. 90		A general plasma stain.	Krause, p. 1293–4.
	Benda.	For spermatozoa; with safranin.	Benda, 1891.
		For cellulose walls in vascular plant tissue; contrast to safranin.	Chamberlain, p. 61.
		For general histological tissues; contrast to hematoxylin.	Peter, 1899. Prenant, 1902. Brazil, 1905.
		Following safranin; in cytology. **See p. 82**	
	Twort.	With neutral red; for parasites in tissues.	Twort, 1924.
Fast green FCF p. 91		As substitute for light green SF yellowish in histology and cytology.	Haynes, 1928.
Basic fuchsin p. 92		A powerful nuclear stain; with various green and blue contrast stains.	Krause, p. 807–9.
		For mucin, fuchsinophile granules; for nuclear elements of central nervous system.	Id.
	Frothingham	Mixed with methylene blue; for staining Negri bodies in nerve cells. **See p. 97**	Mal. & Wr., p. 440.
	MacCallum	Preceding crystal violet; for staining bacilli in tissues. **See p. 107.**	MacCallum, 1919.

*The pages listed in this column refer to the present book.
†The references in this column are to the list in appendix III, p. 204.
‡The pages named in this column in bold faced type refer to the places in this book where the procedures in question are given in detail.

Name of stain and page reference*	Name or author of technic	Application	Bibliographic references†
Basic fuchsin p. 92 (*continued*)	Weigert.	For elastic tissue.	Weigert, 1898. Krause, p. 483.
		General bacterial staining. See p. 96‡.	Mal. & Wr., p. 272.
	Ziehl-Neelson.	For staining tubercle organism; diagnosis by acid-fast property. See p. 96.	Krause, p. 2197. Mal. & Wr., p. 393–8.
	Endo.	In bacteriological culture media for differentiating colon and typhoid organisms. See p. 97	Endo, 1904. Mal. & Wr., p. 227–8.
	Feulgen.	As microchemical reagent for recognition of thymonucleic acid in cell nuclei.	Feulgen and Voit, 1924.
Acid fuchsin p. 97		A widely used plasma stain.	Lee, p. 171, 321.
	Van Gieson.	In combination with picric acid; for connective tissue. See p. 40	Lee, p. 176. Krause, p. 407, 2039. Mal. & Wr., p. 80,119.
	Mallory.	In combination with anilin blue and orange G; for connective tissue. See p. 114	Mallory, 1900. Mal. & Wr., p. 118. Krause, p. 65.
	Ehrlich-Biondi-Heidenhain.	In combination with methyl green and orange G; for differential staining of sections and blood smears.	Krause, p. 457. Lee, p. 173–5.
	Ehrlich's tri-acid mixture.	In combination with methyl green and orange G; for blood smears.	Krause, p. 457, 1707. Lee, p. 175.
		For cortex, pith, cellulose walls, etc., in vascular plants.	Chamberlain, p. 57,67
	Pianese.	For cancer tissue; with malachite green and martius yellow. See p. 41	Pianese, 1896.

		Same technic adapted for staining fungus mycelium in infected plants.	Müller, 1912. Vaughan, 1914.
	Bensley-Cowdry.	For mitochondria; with methyl green. **See p. 99**	Lee, p. 324.
	Andrade.	As indicator; especially in bacteriological media.	Mal. & Wr., p. 238.
Ethyl violet p. 100	Bowie.	In combination with Biebrich scarlet; for staining the islets of Langerhans.	Bowie, 1924.
Hoffman's violet p. 100	Ehrlich.	For mast cells.	Krause, p. 1123.
	Juergens.	For amyloid in sections of tissue.	Id.
Methyl or Gentian violet p. 101		A powerful nuclear stain.	Krause, p. 852.
		A very valuable stain in plant histology, particularly for the achromatic nuclear structures.	Chamberlain, p. 59–60
	Flemming's triple stain.	For nuclear structures in conjunction with safranin; orange G as contrast stain. **See p. 81**	Flemming, 1881. Lee, p. 177. Ehrl. I, p. 475–7. Krause, p. 1736.
	Weigert.	For fibrin and neuroglia in fresh tissue.	Weigert, 1881. Mal. & Wr., p. 150–1
		For amyloid; in frozen sections of fresh tissue.	Mal. & Wr., p. 202.
	Bensley.	Combined with orange G, to form "neutral gentian:" for demonstrating islands of Langerhans.	Bensley, 1911.

*The pages listed in this column refer to the present book.
†The references in this column are to the list in appendix III, p. 204.
‡The pages named in this column in bold faced type refer to the places in this book where the procedures in question are given in detail.

Name of stain and page reference*	Name or author of technic	Application	Bibliographic references†
Crystal violet p. 104	Gram-Weigert.	For staining bacteria in tissues. See p. 104‡	Mal. & Wr., p. 288.
	Gram.	As bacterial stain; generally with alcoholic differentiation to make it selective, and some red or brown dye as counterstain. See p. 106	Gram, 1884. Hucker & Conn, 1923. Benda, 1899.
	Benda.	For mitochondria; with alizarin red. See p. 108	Krause, p. 1562. Lee, p. 322.
	Jackson	For lightly lignified walls in plants; with erythrosin. (Proves more uniform than gentian violet.) See p. 108	Jackson, 1926.
	MacCallum	Following basic fuchsin and picric acid; for staining influenza bacilli in tissues. See p. 107	MacCallum, 1919.
Methyl green p. 109		An excellent nuclear stain.	Lee, p. 159–61. Krause, p. 1416.
		In weak acetic acid solution, for fresh chromatin.	Lee, p. 293–4.
		For lignified xylem in plants; with acid fuchsin.	Chamberlain, p. 61.
	Galeotti.	As cytoplasm stain; following acid fuchsin and picric acid.	Krause, p. 1417.
	Ehrlich-Biondi-Heidenhain.	In combination with acid fuchsin and orange G; stains nuclei in sections and in blood.	Krause, p. 457. Lee, p. 173–5.
	Bensley-Cowdry.	For chromatin; in contrast to acid fuchsin, which stains the mitochondria. See p. 99	Lee, p. 324.
	Ehrlich's tri-acid mixture.	In combination with acid fuchsin and orange G; for blood smears.	Krause, p. 457, 1707. Lee, p. 175.

	Pappenheim.	In combination with pyronin; for gonococcus, and for mast cells. **See p. 119**	Pappenheim, 1899. Krause, p. 1990. Lee, p. 172.
Iodine green p. 110	Griesbach.	As nuclear stain.	Griesbach, 1882.
		For mucin and amyloid.	Stilling, 1886. Krause, p. 59.
	Zimmermann.	For lignified xylem in plant sections; with acid fuchsin.	Chamberlain, p. 61,67
		For chromatin in plant tissue; with acid fuchsin for nucleolus and spindle fibres.	Zimmermann, 1893. Krause, p. 1122.
	Giaccio.	For nervous tissue; with acid fuchsin and picric acid.	Giaccio, 1906.
	Lefas.	For blood; with acid fuchsin.	Krause, p. 1122.
Methyl blue p. 112	Mann.	For nerve cells; with eosin. **See p. 112**	Lee, p. 183. Krause, p. 649.
	Dubreuil.	Combined with picric acid, as tissue stain; followed by carmalum or safranin.	Krause, p. 1382.
Anilin blue, water soluble p. 113		A good plasma stain, used particularly in nervous tissue and cartilage.	Lee, p. 183. Krause, p. 63.
	Mallory.	For connective tissue; with orange G and acid fuchsin. **See p. 114**	Mallory, 1900. Mal. & Wr., p. 118. Krause, p. 63.
	Stroebe-Huber.	As cytoplasm stain, preceding safranin.	Krause, p. 63.
	Unna.	For sections of epithelium; with orcein.	Lee, p. 340.
Rosolic acid p. 115	Pappenheim.	With methylene blue in a decolorizing solution following basic fuchsin; for tubercle organism. **See p. 116**	Pappenheim, 1898.

*The pages listed in this column refer to the present book.
†The references in this column are to the list in appendix III, p. 204.
‡The pages named in this column in bold faced type refer to the places in this book where the procedures in question are given in detail.

Name of stain and page reference*	Name or author of technic	Application	Bibliographic references†
Pyronin p. 118	Pappenheim-Saathof .	In combination with methyl green; for bacteria (especially in organic liquids), mast cells and other basophile elements. See p. 119‡	Pappenheim, 1899. Lee, p. 172. Krause, p. 1990.
	Ehrlich.	In combination with narcein and methyl green or methylene blue, for blood, etc.	Ehrlich and Lazarus, 1898.
		For bacteria; sometimes used as counterstain to gentian violet in the Gram technic.	Mal. & Wr., p. 273.
Rhodamine B or S p. 119	Griesbach.	With osmic acid; to fix and stain blood simultaneously.	Krause, p. 2023.
		As plasma stain, in contrast to methyl green or methylene blue.	Id.
	Ehrlich.	As a component of certain "neutral" stain mixtures; for blood, etc.	Id.
Eosin Y p. 123		Very usful cytoplasm stain. See p. 124	Krause, p. 646–52.
		For algae and fungi; counterstain to hematoxylin, etc.	Chamberlain, p. 57–8.
	Mann.	In combination with methyl blue; for sections of tissues. See p. 112	Krause, p. 649.
	List.	Cytoplasm stain; preceding methyl green.	List, 1885.
	Teichmüller.	For sputum; preceding methylene blue.	Teichmüller, 1899.
	Romanovsky, etc.	In combination with methylene blue; for blood smears. See p. 144-8	Krause, p. 188–9. Mal. & Wr., p. 470–8.
Ethyl eosin p. 125		Counterstain following Delafield's hematoxylin in general animal histology.	
	Harris	Stain for Negri bodies in smears of nervous tissue. See p. 126	Harris, 1908
		Stain for Negri bodies in sections of nervous tissue. See p. 126	

Eosin, bluish p. 127		Counterstain, especially following Mayer's hemalum.	
Mercurochrome 220 p. 127		Substitute for eosin.	Baldwin, 1928.
		Fungicidal agent for treating amphibian embryos.	Detwiler, 1928.
Erythrosin p. 128	Held.	Cytoplasm stain for nerve cells; preceding methylene blue. See p. 69	Held, 1895.
		Counterstain for sections of vascular plants; in contrast to hematoxylin, gentian violet, etc.	Chamberlain, p. 58.
	Winogradsky.	Stain for bacteria in dried smears of soil suspensions. See p. 129	Winogradsky, 1924.
	Jackson	Counterstain to crystal violet in plant histology. See p. 108	Jackson, 1926.
Phloxine p. 130	Chamberlain.	For staining algae. See p. 131	Chamberlain, p. 59.
	Unna.	Combined with various other acid dyes; for use in studying chromolysis.	Unna, 1921.
	Mallory.	As cytoplasm stain in contrast to methylene blue, on pathological or other histological material. See p. 131	Mallory, 1904. Mal. & Wr., p. 100–2.
Rose Bengal p. 132		Cytoplasm stain, following hematoxylin.	Pelagetti, 1904.
	Conn.	Stain for bacteria, especially those forming slime; also for dried smears of soil suspensions. See p. 133	Conn, 1921.
Indigo carmin p. 150		As plasma stain in contrast to carmin, either with it or following it.	Lee, p. 212. Krause, p. 1045.

*The pages listed in this column refer to the present book.
†The references in this column are to the list in appendix III, p. 204.
‡The pages named in this column in bold faced type refer to the places in this book where the procedures in question are given in detail.

Name of stain, mordant, etc. Also page ref.*	Name or author of technic	Application	Bibliographic references†
Cochineal, p. 139			For general discussion see Krause, p. 394–8.
Alone, aqueous.		Only for objects containing iron, aluminium, etc.	
Alone, alcoholic.	Mayer's old formula	Especially for arthropod tissue.	Lee, p. 143.
Tincture, (with $CaCl_2$ & $AlCl_3$.)	Mayer.	For sections and staining in bulk. Used like paracarmin (see below).	Mayer, 1878. Mayer, 1892. Lee, p. 144.
Alum-cochineal.	Mayer. Czokor. Partsch.	For staining in bulk; not suited for sections. (Stains nuclei violet red, blood and muscle cells orange; colors cytoplasm weakly.)	Mayer, 1878. Mayer, 1892. Czokor, 1880. Chamberlain, p. 53.
Chromalum cochineal.	Hansen.	For sections.	Hansen, 1905.
Iron alum cochineal (i. e. following iron alum).	Spuler.	For staining in bulk for photographing; also for sections; brings out nuclei, blood in tissues, and muscle striations.	Spuler, 1901. Lee, p. 140.
Ferri-cochineal (i. e. with iron alum.)	Hansen.	For sections	Lee, p. 140.
Carmin, p. 151		For staining by injection and for bulk staining. Valuable in embryology.	For general discussion see Krause, p. 260–8.
Aceto-carmin	Schneider.	As nuclear stain for sections; for fresh chromosomes in smear preparations.	Lee, p. 138.

Iron-aceto carmin.	Belling.	Stain for chromosomes in smears of anthers. **See p. 153‡**	Belling, 1921.
Ammonia carmin	Ranvier. Hoyer.	For sections.	Lee, p. 140.
	Van Vijhe.	For injection.	Id.
	Best.	For staining glycogen in sections. **See p. 153**	Best, 1906.
Magnesia carmin	Mayer.	For sections, and for bulk staining.	Mayer, 1892. Mayer, 1896. Lee, p. 140.
Borax carmin (alcoholic).	Grenacher. Mayer.	A much used stain for sections. **See p. 152**	Mayer, 1892, 1896. Lee, p. 141. Chamberlain, p. 52.
Hydrochloric carmin (alcoholic).	Mayer.	For sections, and for bulk staining.	Mayer, 1892, 1896.
Alum carmin.	Grenacher.	As nuclear stain for sections.	Grenacher, 1879. Lee, p. 136–8.
Lithium carmin.	Orth.	As nuclear stain for sections.	Krause p. 265.
With $AlCl_3$. "Muci-carmin"	Mayer.	For mucin, in sections.	Mayer, 1892, 1896.
Iron carmin.		For sections.	Lee, p. 139.
Picro-carmin (with picric acid).		For double staining effect in sections, particularly of nervous tissue. Nuclei red; cytoplasm yellow.	Lee, p. 140–1.

*The pages listed in this column refer to the present book.
†The references in this column are to the list in appendix III, p. 204.
‡The pages named in this column in bold faced type refer to the places in this book where the procedures in question are given in detail.

Name of stain, mordant, etc. Also page ref.*	Name or author of technic	Application	Bibliographic references†
Carminic acid, p. 151			For general discussion see Krause, p. 268–71.
Carmalum (with alum).	Mayer.	As nuclear stain for sections. See p. 152‡	Mayer, 1892, 1899. Lee, p. 137. Chamberlain, p. 53.
	Rawitz.	Prepared with glycerin; for sections.	Rawitz, 1899. Lee, p. 138.
Muci-carminic acid (acid, with $AlCl_3$.)	Rawitz.	For mucin, in sections.	Rawitz. 1899.
Para-carmin (with $AlCl_3$ and $CaCl_2$).	Mayer.	For sections and staining in bulk; ordinarily stains cytoplasm as well as nuclei.	Mayer, 1892, 1899. Lee, p. 142.
With iron.	Weigert. v. Wellheim.	Following treatment with iron chloride; for sections and staining in bulk.	Lee, p. 139.
	Zacharias.	Citrate of iron, following an aceto-carmin.	Lee, p. 139.
	Unna.	In alcoholic solution; for elastic tissue.	Mal. & Wr., p 123. Krause, p. 483, 1738. Lee, p. 353.
	Israel.	Dissolved in acetic acid solution. Stains nuclei blue, cytoplasm red.	Lee, p. 212. Krause, p. 1737.
Orcein, p. 153	Moll.	In HCl solution; for sections of embryos.	Lee, p. 376. Krause, p. 1737.

	Unna.	For connective tissue; following polychrome methylene blue.	Lee, p. 161. Krause, p. 485, 173
	Unna.	For plasma fibrils of epithelium; following anilin blue.	Lee, p. 161.
Brazilin, p. 154	Mayer.	With alum, as a nuclear stain (like hemalum). Called brazilum.	Lee and Mayer, 1907, p. 218. Lee, p. 211. Krause, p. 247.
	Hickson.	As nuclear stain, following iron alum.	Hickson, 1901. Lee, p. 211. Krause, p. 246.
	Belling.	For staining chromosomes in smears of anthers.	Belling, 1928.
Hematoxylin and hematein, p. 155			For general discussio see Krause, p. 958–9
Alone.		As nuclear stain for plant sections.	Krause, p. 966.
		As reagent for iron and copper.	Id.
With aluminium (alum-hematoxylin).	Böhmer.	Obsolete tissue stain; historic interest only.	Böhmer, 1865.
	Delafield.	As nuclear stain for tissues. For cellulose walls of plants. See p. 158	Lee, p. 151–5. Chamberlain, p. 48–5
	Ehrlich.	As nuclear stain for tissues. See p. 158	Krause, p. 972.
Hemalum (hematein with alum)	Mayer, and later modifications.	As nuclear stain for tissues. See p. 159	Mayer, 1891, 1899. Lee, p. 152. Krause, p. 971.

*The pages listed in this column refer to the present book.
†The references in this column are to the list in appendix III, p. 204.
‡The pages named in this column in bold faced type refer to the places in this book where the procedures in question are given in detail

Name of stain, mordant, etc. Also page reference*	Name or author of technic	Application	Bibliographic references†
Hemacalcium (hematein with $AlCl_3$ and $CaCl_2$).	Mayer.	As nuclear stain for tissues.	Mayer, 1891, 1899. Lee, p. 155. Krause, p. 972.
Muc-hematein (with $AlCl_3$ and glycerin).	Mayer.	As stain for mucin.	Mayer, 1891, 1899. Krause, p. 979.
With chromium.	Heidenhain, R.	An early technic for tissue staining; still used.	Chamberlain, p. 44–8.
	Apathy. Hennegui. Hansen. Schultz.	Recent modifications; for general tissue staining.	Lee, p. 156–7. Krause, p. 989–90.
	Weigert.	For nervous tissue.	Weigert, 1884. Krause, p. 1019.
Following an iron salt.	Benda.	An obsolete technic; the original method.	Krause, p. 978.
	Heidenhain, M.	One of the most useful present histological and cytological stains, both in botany and zoology. See p. 159‡	Lee, p. 157–62. Krause, p. 974.
	Weigert.	A valuable nuclear stain for sections. See p. 159	Mal. & Wr., p. 95
	Butschli. Hansen. Kaiser.	Modifications for various special purposes.	Rhrl. 1, p. 607. Krause, p. 975–88.

Hematoxylin (Continued)

Differentiating Stains

Hematoxylin (Continued)

Following copper.	Benda.	For studying spermatogenesis.	Krause, p. 990.
	Bensley.	For chromosomes and mitochondria.	Guyer, 1917, p. 145.
With phosphomolybdic acid.	Mallory.	For central nervous system.	Mal. & Wr., p. 71. Lee, p. 157. Krause, p. 991.
With phosphotungstic acid.	Mallory.	A very valuable pathological stain, especially for fibrils in tumor tissue. See p. 159	Mal. & Wr., p. 72. Lee, p. 158. Krause, p. 992.
With vanadium.	Heidenhain, M.	For general tissue.	Ehrl. I, p. 604. Lee, p. 157. Krause, p. 993.
Double staining.	Van Gieson.	Followed by picric acid and acid fuchsin. See p. 40 Sometimes followed by picric acid, or following eosin, orange G, safranin or acid fuchsin.	Lee, p. 176.

*The pages listed in this column refer to the present book.
†The references in this column are to the list in appendix III, p. 204.
‡The pages named in this column in bold faced type refer to the places in this book where the procedures in question are given in detail.

TABLE 3.—LIST OF BIOLOGICAL STAINS OR DYES ARRANGED ALPHABETICALLY.

Aniline blue water soluble
Bismarck brown Y
Brilliant cresyl blue
Brilliant green
Carmin
Congo red
Cresyl violet
Crystal violet
Eosin, bluish
Eosin, yellowish
Ethyl eosin
Fast green FCF
Fuchsin, acid
Fuchsin, basic
Hematoxylin
Indigo carmin
Janus green B
Jenner's stain
Light green S. F. yellowish
Malachite green
Martius yellow
Methyl blue
Methyl green
Methyl violet
Methylene blue
Methylene violet
Neutral red
Nile blue A
Nigrosin
Orange G
Orange II
Phloxine
Pyronin
Rose bengal
Safranin O
Sudan III
Sudan IV
Tetrachrome stain (MacNeal)
Thionin
Toluidine blue
Wright's stain

TABLE 4. LIST OF BIOLOGICAL STAINS GROUPED ACCORDING TO THE FIELD IN WHICH USED.*

ANIMAL HISTOLOGY

Nuclear stains (basic)

Janus green B.
Thionin
Azure A
Azure C
Methylene blue
Toluidine blue
Cresyl violet
Safranin
Magdala red
Auramin
Fuchsin
Hoffman's violet
Iodine green
Gentian violet (including crystal and methyl violets)
Cochineal and carmin
Orcein
Brasilin
Hematoxylin

Cytoplasm stains (acid)

Picric acid
Orange G.
Bordeaux red
Fast yellow (bone tissue)
Methyl orange (for keratin in skin)
Amaranth (nervous tissue)
Biebrich scarlet W. S.
Bismarck brown
Chrysoidin Y
Congo red
Benzopurpurin 4B
Alizarin red S (for nervous tissue)
Nigrosin
Malachite green
Light green SF yellowish
Fast green FCF
Acid fuchsin
Methyl blue
Aniline blue W. S.
Rhodamine
Eosin Y.
Ethyl eosin
Phloxine
Erythrosin (various types)
Indigo carmin

Fat stains
- Sudan III
- Sudan IV
- Oil red O
- Nile blue sulfate

Vital stains
- Bismarck brown
- Chrysoidin Y
- Trypan red
- Benzopurpurin 4B
- Trypan blue
- Vital red
- Dianil blue 2B
- Methylene blue
- Thionin
- Toluidine blue
- Nile blue sulfate
- Brilliant cresyl blue
- Neutral red
- Safranin O
- Janus green B
- Crystal violet
- Methyl violet

PLANT HISTOLOGY

Nuclear stains (basic)
- Methylene green (wood and fixed chromatin)
- Safranin
- Magdala red
- Auramin
- Iodine green
- Gentian violet (including crystal and methyl violets)
- Methyl green (lignified xylem)
- Cochineal and carmin
- Hematoxylin

Cytoplasm stains (acid)
- Martius yellow (for host tissue in case of fungus diseases)
- Orange G.
- Congo red (plant mucin)
- Nigrosin
- Malachite green (for host tissue in case of fungus diseases)
- Light green SF yellowish (cellulose walls)
- Fast green FCF
- Acid fuchsin
- Eosin Y
- Erythrosin.

CYTOLOGY

General nuclear stains (basic)
- Thionin
- Methylene blue
- Toluidine blue
- Magdala red
- Fuchsin
- Gentian violet (including crystal and methyl violets)
- Methyl green
- Carmin
- Orcein
- Hematoxylin

Special chromatin stains
- Alizarin red S
- Thionin
- Methylene green
- Safranin
- Iodine green
- Hematoxylin
- Crystal violet

Cytoplasm stains (acid)
- Picric acid
- Orange G.
- Light green SF yellowish
- Fast green FCF
- Bordeaux red
- Methyl orange
- Acid fuchsin
- Eosin Y.

Stains for cell inclusions (mitochondria, etc.)
- Aurantia
- Janus green B.
- Acid fuchsin
- Crystal violet.

Nuclear stains (basic)

- **Thionin**
- **Azure A**
- **Azure C**
- **Methylene blue**
- **Toluidine blue**
- **Safranin**
- **Cresyl violet**
- **Fuchsin**
- **Hoffman's violet**
- **Iodine green**
- **Gentian violet (including crystal and methyl violets)**
- **Pyronin**
- **Cochineal and carmin**
- **Orcein**
- **Hematoxylin**

Cytoplasm stains (acid)

- **Picric acid**
- **Martius yellow (for cancer tissue)**
- **Orange G.**
- **Methyl orange**
- **Amaranth (for nervous tissue)**
- **Biebrich scarlet W. S.**
- **Congo red**
- **Alizarin red S.**
- **Neutral red**
- **Nigrosin**
- **Malachite green**
- **Light green SF yellowish**
- **Acid fuchsin**
- **Methyl blue**
- **Aniline blue, W. S.**
- **Eosin Y.**
- **Ethyl eosin**
- **Phloxine**
- **Erythrosin**

Blood stain constituents

- **Orange G (acid)**
- **Narcein (acid)**
- **Methylene blue (basic)**
- **Azure A (basic)**
- **Neutral red (acid)**
- **Acid fuchsin (acid)**
- **Methyl green (basic)**
- **Pyronin (basic)**
- **Rhodamine (basic)**
- **Eosin Y (acid)**

Fat stains

- **Sudan IV**
- **Nile blue sulfate.**

Bacterial stains

- **Bismarck brown (Gram counterstain)**
- **Thionin**
- **Methylene blue**
- **Safranin (Gram counterstain)**
- **Fuchsin**
- **Gentian violet (including crystal and methyl violets)**
- **Methyl green (constituent of Pappenheim stain)**
- **Pyronin**
- **Erythrosin**
- **Rose bengal**

Used in bacteriological media

- **Neutral red**
- **Fuchsin**
- **Acid fuchsin**
- **Brilliant green**
- **Methylene blue**
- **Eosin Y**

TABLE 5. DYE SOLUBILITIES AT 26°C.

Based on data obtained at the Color Laboratory of the U. S. Dept. of Agriculture.

See Holmes, (1927, 1928, 1929)

C. I. Number	Name of dye	Per cent soluble in	
		Water	95% alcohol
7	Picric acid	1.18	8.96
8	Victoria yellow	1.66	1.18
9	Martius yellow, Na salt	4.57	0.16
	Martius yellow, Ca salt	0.05	1.90
10	Naphthol yellow G	8.96	0.025
12	Aurantia	nil	0.33
16	Fast yellow	18.40	0.24
20	Chrysoidin Y	0.86	2.21
21	Chrysoidin R	0.23	0.99
24	Sudan I	nil	0.37
27	Orange G	10.86	0.22
28	Ponceau 2G	1.75	0.21
29	Chromotrope 2R	19.30	0.17
36	Alizarole yellow GW	25.84	0.04
40	Alizarole orange G	0.40	0.57
73	Oil red O	nil	0.39
88	Azo bordeaux	3.83	0.19
89	Crystal ponceau	0.80	0.06
130	Erika B	0.64	0.17
138	Janus green	5.18	1.12
138	Metanil yellow	5.36	1.45
142	Methyl orange	0.52	0.08
	Methyl orange (acid)	0.015	0.015
146	Azo acid yellow	2.17	0.81
148	Resorcin yellow	0.37	0.19
150	Orange I	5.17	0.64
151	Orange II	11.37	0.15
152	Narcein	10.02	0.06
176	Fast red A	1.67	0.42
184	Amaranth	7.20	0.01
186	Ponceau 6R	12.98	0.01
248	Sudan III	nil	0.15
252	Brilliant croceine	5.04	0.06
254	Erythine X	6.41	0.06
258	Sudan IV	nil	0.09
280	Biebrich scarlet	—	0.05
331	Bismarck brown Y	1.36	1.08
332	Bismarck brown R	1.10	0.98
370	Congo red	—	0.19
448	Benzo purpurin 4B	—	0.13
520	Niagara blue 4B	13.51	nil
1027	Alizarin	nil	0.125
1034	Alizarin red S	7.69	0.15
920	Thionin	0.25	0.25
922	Methylene blue (ZnCl$_2$ double salt)	2.75	0.05
	Methylene blue (chloride)	3.55	1.48
	Methylene blue (iodide)	0.09	0.13
924	Methylene green	1.46	0.12

C. I. Number	Name of dye	Per cent solubility at 25° C.	
		Water	95% alcohol
925	Toluidine blue O	3.93	0.62
927	New methylene blue N	19.82	1.85
845	Neutral red (chloride)	5.64	2.15
	Neutral red (iodide)	0.12	0.16
826	Neutral violet	3.27	2.45
841	Safranin	5.45	3.41
847	Amethyst violet	3.13	5.66
914	Nile blue 2B	0.16	0.62
——	Cresyl violet (N. A. Co.)	0.38	0.25
655	Auramin O	0.74	4.19
657	Malachite green (oxalate)	7.60	7.52
659	Victoria green 3B	0.04	2.24
666	Guinea green B	28.40*	7.30
670	Light green SF yellowish	20.35	0.92
——	Fast green FCF	18.04	0.35
676	Pararosanilin (chloride)	0.26	5.93
——	Pararosanilin (acetate)	4.15	13.63
——	Rosanilin (chloride)	0.20	8.16
678	New fuchsin (chloride)	1.13	3.20
680	Methyl violet	2.95	15.21*
681	Crystal violet (chloride)	1.68	13.87
	Crystal violet (iodide)	0.035	1.78*
689	Spirit blue	nil	1.10
690	Victoria blue 4R	3.25	20.49
714	Patent blue A	8.40	5.23
715	Cyanole extra	1.38	0.44
723	New Victoria blue R	0.54	3.98
739	Pyronin G	8.96	0.60
741	Pyronin B (iodide)	0.07	1.06
749	Rhodamine B	0.78	1.47
750	Rhodamine G	1.34	6.31
766	Fluorescein (color acid)	0.03	2.21
	Fluorescein (Na salt)	50.20	7.19
	Fluorescein (Mg salt)	4.51	0.35
	Fluorescein (Ca salt)	1.13	0.41
	Flurescein (Ba salt)	6.54	0.56
768	Eosin Y† (Na salt)	44.20	2.18
	Eosin Y† (Mg salt)	1.43	0.33
	Eosin Y† (Ca salt)	0.24	0.09
	Eosin Y† (Ba salt)	0.18	0.09
770	Ethyl eosin	0.08	1.13
773	Eosin B (Na salt)	39.11	0.75
775	Erythrosin† (Na salt)	11.10	1.87
	Erythrosin† (Mg salt)	0.59	0.52
	Erythrosin† (Ca salt)	0.15	0.35
	Erythrosin† (Ba salt)	0.17	0.04

*These figures are grams per hundred grams of saturated solution (the others being grams per hundred cubic centimeters).

†The color acids of these dyes (not listed here) are practically insoluble in water.

TABLE 8—Continued

C.I. Number	Name of dye	Per cent soluble in	
		Water	95% alcohol
774	Phloxine† (Na salt)	50.90*	9.02
	Phloxine† (Mg salt)	20.84	29.10
	Phloxine †(Ca salt)	3.57	0.45
	Phloxine† (Ba salt)	6.01	1.17
779	Rose bengal† (Na salt)	36.25	7.53
	Rose bengal† (Mg salt)	0.48	1.59
	Rose bengal† (Ca salt)	0.20	0.07
	Rose bengal† (Ba salt)	0.17	0.05
1180	Indigo carmin	1.68	0.01

*These figures are grams per hundred grams of saturated solution (the others being grams per hundred cubic centimeters).

†The color acids of these dyes (not listed here) are practically insoluble in water.

APPENDIX II

GENERAL LABORATORY INFORMATION

1. Solubility of Miscellaneous Compounds Used in Microscopic Work.*

	Per cent soluble in				
	Water				Alcohol
	20°C	25°C	30°C	100°C	
Ammonia alum	15.13	19.19	22.01	357.00	Insoluble
Copper sulfate ($5H_2O$)	42.31		48.81	203.3	Insoluble
Ferric chloride	74 or more				Quite soluble
Lithium carbonate	1.33			0.728	Insoluble
Mercuric chloride	7.39		8.43	53.9	49.5 at 25°
Potassium alum	11.40	14.14	16.58	422.00	
Potassium dichromate	12 to 13		18.13	102.00	Insoluble
Sodium sulfate ($10\ H_2O$)	58.85	98.48	184.1	312.00	Insoluble
Urea	104.7		136.0		5.4 at 20°
Anilin	3.11 at 16°				
Tannic acid	20 to 300				50 to 400

2. Formulae of Fixing Solutions

1) Formol:
   Formalin (40% formaldehyde) ..... 4–10 parts
   Water ..... 100 parts

2) Mercuric chloride:
   Sat. Aqu. (or Alc.) Sol. mercuric chloride
   5% Glacial acetic acid.

The proportions of these two solutions to mix together must be determined experimentally in any instance, employing enough of the acetic acid to overcome the shrinking action of the sublimate.

3) Gilson's fluid.
   Nitric acid, 46° strength (about an 80% solution) ..... 15 cc.
   Glacial acetic acid ..... 4 cc.
   60% alcohol ..... 100 cc.
   Distilled water ..... 880 cc.
   Mercuric chloride ..... 20 g.

4) Sublimate acetic:
   Sat. Aqu. Sol. of mercuric chloride ..... 95 parts
   Glacial acetic acid ..... 5 parts

5) Mueller's fluid:
   Potassium bichromate ..... 25 g.
   Sodium sulfate ..... 10 g.
   Water ..... 1000 cc.

*The data given in this table were obtained from various sources in the literature, and their accuracy is not vouched for; they are, however, sufficiently correct to guide anyone in making up a saturated solution of any of the above compounds.

6) Zenker's fluid:

Potassium bichromate	25 g.
Mercuric chloride	5–8 g.
Water	ad 100 cc.
Glacial acetic acid	5 cc.

Dissolve the mercuric chloride and potassium bichromate in the water with the aid of heat. Do not add the acetic acid to the stock solution, but only to the part taken for hardening pieces of tissue.

7) Helly's fluid, or Zenker-formol:
This is a modification of Zenker in which the acetic acid is replaced by 5% formalin.

8) Carnoy's fluid:

Glacial acetic acid	1 part
Absolute alcohol	6 parts
Chloroform	3 parts

9) Formol-nitric:

Formol, 10%	3 parts
Nitric acid, 10%	1 part

10) Flemming's fluid. Strong formula.

(a)	Chromic acid, 1% Aqu. Sol.	11 parts
	Glacial acetic acid	1 part
	Distilled water	4 parts

(b) Osmic acid, 2% in 1% chromic acid solution.

Just before using mix 4 parts of (a) with one part of (b). Use 10 times the volume of the object.

11) Bichromate-chromic-osmic acid mixture of Champy:

3% Aqu. Sol. of bichromate of potash	7 parts
1% chromic acid Aqu. Sol.	7 parts
Osmic acid, 2% Aqu. Sol.	4 parts

12) Platino-aceto-osmic acid mixture of Hermann:

Platinic chloride, 1% Aqu. Sol.	15 parts
Glacial acetic acid	1 part
Osmic acid, 2% Aqu. Sol.	2 parts

13) Merkel's fluid (F. E. V. Smith's modification):

Acetic acid, 5% Aqu. Sol.	100 cc.
Platinic chloride, 1% Aqu. Sol.	5 cc.
Chromic acid, 1% Aqu. Sol.	10 cc.

14) Bouin's fluid:

Sat. Aqu. Sol. picric acid	75 parts
Formol, C. P.	25 parts
Acetic acid, glacial	5 parts

15) Allen's fluid P. F. A_3:

Picric acid, Sat. Aqu. Sol.	75 parts
Formalin, C. P.	15 parts
Glacial acetic acid	10 parts
Urea	1 part

3. Formula of Cleaning Fluid For Slides, Cover Glasses, Etc.

Potassium or sodium bichromate (commercial grade satisfactory).	40 g.
Water	150 cc.

Dissolve with a little heat if necessary, then cool to room temperature and add the following slowly:

Concentrated sulfuric acid	230 cc.

APPENDIX III

BIBLIOGRAPHY

(Matter in parenthesis indicates the purpose for which each reference is cited in the preceding pages, not necessarily the main subject matter of the article in question.)

ADAMS, A. ELIZABETH.

1928. Paraffin sections of tissue supra-vitally stained. *Sci.*, **68**, *303–4*. (Employs nile blue sulfate).

ANONYMOUS.

1865. Injectionsmassen von Thiersch und Müller, *Arch. Mikr. Anat.*, **1**, *148*. (Use of carminates with oxalic acid.)

ALBERT, H.

1920. Diphtheria bacillus stains with a description of a "new" one. *Am. J. Pub. H.*, **10**, *334*. (Toluidine blue for staining diphtheria preparations.)

AMBLER, J. A., and HOLMES, W. C.

1924. The investigation of biological stains in the Color Laboratory of the Bureau of Chemistry. *Sci.*, **60**, *501–502*.

BALL, G. H.

1926. Studies on Paramecium: III. The effects of vital dyes on *Paramecium caudatum*. *Biol. Bull.*, **52**, *68–78*.

BAILEY, P.

1921. Cytological observations on the pars buccalis of the hypophysis cerebri of man, normal and pathological. *J. Med. Res.*, **42**, *349–381*. (Employs acid violet as a counterstain to Altmann's anilin fuchsin, also alone, and in neutral combination with safranin.)

BALDWIN, WESLEY MANNING.

1928. Mercurochrome-220 soluble as a histologic stain. *Anat. Rec.*, **39**, *229*.

BELLING, JOHN.

1921. On counting chromosomes in pollen mother cells. *Amer. Nat.*, **55**, *573–4*. (Stains with iron-aceto-carmin.)

1928. A method for the study of chromosomes in pollen mother cells. *U. of Cal., Pub. in Bot.*, **14**, *No. 9, 293–299*. (Use of brazilin for staining chromosomes in pollen-mother-cells.)

BENDA, CARL.

1891. Neue Mittheilungen über die Entwickelung der Genitaldrüsen and über die Metamorphose der Samenzellen. *Arch. f. Anat. u. Phys.* (*Phys. Abt.*) *1891, 549–552*. (Safranin with light green for staining spermatozoa.)

1899. Weitere Mittheilungen über die Mitochondria. *Arch. f. Anat. u. Phys.* (*Phys. Abt.*) *1899, 376–383*. (Proposes crystal-violet-alizarin method for chondriosomes.)

1901. Die Mitochondriafärbung und andere Methoden zur untersuchung der Zellsubstanzen. *Anat. Anz., Ergänzhft.* **19**, *155–174*. (Describes the crystal-violet-alizarin method.)

BENEKE.

1862. *Correspbl. d. Ver. f. gemeinsch. Arbeiten, No. 59, 980*. (A note without title, being first reference to use of anilin dyes in histology.)

BENSLEY, R. R.

1911. Studies on the pancreas of the guinea pig. *Am. J. Anat.*, **12**, *297–388*. (Acid fuchsin and Janus green for chondriosomes. Describes "neutral gentian.")

BERGONZINI, C.

1891. Über das Vorkommen von granulierten basophilen und acidophilen Zellen im Bindegewebe und über die Art sie sichtbar zu machen.

Anat. Anz., 6, *595–600.* (Methyl orange in place of orange G in Ehrlich-Biondi stain.)

BERNTHSEN, A.
1885. Studien in der Methylen blau gruppe. *Liebig's Ann. de. Chimie.*, 230, *73–136, 137–211.* (Chemistry of Azur I, etc.)
1906. Ueber die chemische Natur des Methylenazurs. *Ber. d. Deut. Chem Gesell.*, 39, II, *1804–1809.*

BEST, F.
1906. Ueber Karminfärbung des Glycogens und der Kerne. *Zts. Wis. Mikr.*, 23, *319–322.*

BLACKMAN, V. H.
1905. Congo red as a stain for Uredineae. *New Phytol.*, 4, *173–4.*

BÖHMER, F.
1865. Zur pathologischen anatomie der Meningitis cerebromedullaris epidemica. *Aerztl. Intelligenzb.* (*Munich*), 12, *539–550.* (First use of alum hematoxylin.)

BÖTTCHER, A.
1869. Ueber Entwickelung und Bau des Gehörlabyrinths nach untersuchungen an Säugethieren. I Theil. *Verh. Kais. Leop.-Carol. deut. Akad. Naturf., Dresden*, 35, *Abh. No. 5, pp. 1–203.* (First use of alcohol for differentiation after staining.)

BOWIE, D. J.
1924. Cytological studies of the islets of Langerhans in a teleost, *Meomaenis griseus. Anat. Rec.*, 29, *57.* (Uses ethyl violet in a neutral stain combination with biebrich scarlet).

BRASIL, LOUIS.
1905. Sur la reproduction des Grégarines monocystidées. *Arch. de Zool. Exper. et Gen.*, 4 *Ser.*, 4, *69–100* (Light green with hematoxylin for sections of seminal vesicles.)

BREED, R. S. and BREW, J. D.
1916. Counting bacteria under the microscope. N. Y. Agr. Exp. Sta., Tech. Bul. 49.

BROWNING, C. H., GILMORE, M., and MACKLE, T. J.
1913. The isolation of typhoid bacilli from feces by means of brilliant green in fluid media. *J. Hyg.*, 13, *335–342.*

BUCKMAN, T. E., and HALLISEY, J. E.
1921. Studies in the properties of blood platelets. *J. Am. Med. Assn.*, 76, *427.* (Crystal violet as a substitute for brilliant cresyl blue in staining platelets.)

BUGNON, M. P.
1919. Sur une nouvelle méthode de coloration élective des membranes végétales lignifiées. *C. R. Acad. Sci.*, 168, *62–64.* (Light green and sudan III for the staining of lignified tissue in distinction from suberized and cutinized tissue).

CARNOY, J. B., and LEBRUN, H.
1897. La fecondation chez l'Ascaris megalocephala. *La Cellule*, 13, *63–195.* (Congo red with hematoxylin and other nuclear stains.)

CHAMBERLAIN, C. J.
1924. Methods in Plant Histology. *Fourth edition. xi and 349. pp. Univ. o Chicago Press.*
1927. Staining with phloxine. *Stain Techn.*, 2, *91–93.* (Recommends phloxine, rather than magdala red, for staining algae.)

CHURCHMAN, J. W.
1927. The structure of *B. anthracis* and reversal of the Gram reaction. *J. Exp. Med.*, 46, *1009–1029.* (Employs gentian violet mixed with acriflavine—"acri-violet"—for its bacteriostatic properties.)

CIACCIO, C.
1906. Rapporti istogenetici tra il simpatico e le cellule cromaffini. *Archivio Ital. di. Anat. e. d. Embriol.*, 5, *256–267.* (Iodine green with acid fuchsin and picric acid for nervous tissue.)

CLARK, W. M., and LUBS, H. A.
1917. The colorimetric determination of hydrogen-ion concentration. *Bact.*, **2**, *1–34, 109–136, 191–236.*

COHEN, B.
1923. Some new sulfonphthalein indicators. A preliminary note. *Public Health Repts.*, **38**, *199.*
1926. Indicator properties of some new sulfonphthaleins. *Public Health Repts.*, **41**, *3051–3074.*

CONN, H. J.
1918. The microscopic study of bacteria in soil. *N. Y. Agr. Exp. Sta., Tech. Bul. 64.*
1921. Rose bengal as a general bacterial stain. *J. Bact.*, **6**, *253–254.*
1926. Recent information concerning basic fuchsin. *Stain Techn.*, **1**, *55–59.*
1927 a. Commission specifications of certain stains. *Stain Techn.*, **2**, *27.*
1927 b. Solutions of MacNeal's tetrachrome stain. *Stain Techn.* **2**, *31.*
1927 c. The hematoxylin problem. *Stain Techn.* **2**, *1–3.*
1929. Note on Loeffler's methylene blue. *Stain Techn.*, **4**, *27.*

CONN, H. J. and HOLMES, W. C.
1926. Fluorescein dyes as bacterial stains: with special reference to their use for soil preparations. *Stain Techn.*, **1**, *87–95.*
1928. Certain factors influencing the staining properties of fluorescein derivatives. *Stain Techn.*, **3**, *94–104.*

CORNER, G. W., and HURNI, F. H.
1918. The non-effect of corpus luteum preparations on the ovulation cycle of the rat. *Amer. J. Phys.*, **46**, *483–486.* (Use of dianil blue 2R as vital stain.)

CORTI, A.
1851. Recherches sur l'organe de l'ouie des mammifères. *Zts. Wis. Zool.*, 3, *109–169.* (An early use of carmin in histology, see note 10, p. 143–4).

COWDRY, E. V.
1928. Results secured by applying the Feulgen reaction to fibroblasts and sarcomatous cells in tissue cultures. *Sci.*, **68**, *138–140.*

CYON, E.
1868. Ueber die Nerven des Peritoneum. *Ber. d. k. Säcks Gesel, d. Wiss. zu Leipzig.*, **20**, *121–127.* (Gives technic of carmin staining.)

CZOKOR, J.
1880. Die Cochenille-Carmin lösung. *Arch. Mik. Anat.*, **18**, *412–414.* (Uses alum cochineal.)

DADDI, L.
1896. Nouvelle méthode pour colorer la graisse dans les tissues. *Archives Ital. de Biol.*, **26**, *143–146.* (Proposes Sudan III.)

DAWSON, A. B., EVANS, H. M., and WHIPPLE, G. H.
1920 Blood volume studies: III. Behavior of a large series of dyes introduced into the circulating blood. *Amer. J. Physiol.*, **51**, *232–256.* (Information as to the value of 62 dyes for vital staining.)

DETWILER, S. R.
1917. On the use of nile blue sulfate in embryonic tissue transplantation. *Anat. Rec.*, **13**, *493.* (Nile blue sulfate as vital stain for embryos.)

DETWILER, S. R., and MCKENNON, G. E.
1929. Mercurochrome (di-brom oxy mercuri fluorescin) as a fungicidal agent in the growth of amphibian embryos. *Anat. Rec.*, **41**, *205.*

v. DIGRALSKI, W., and CONRADI, H.
1902. Ueber ein Verfahren zum Nachweis der typhus Bazillen. *Zts. f. Hyg.* **39**, *283.* (Uses crystal violet in agar.)

DORNER, W.
1926. Un procédé simple pour la coloration des spores. *Le Lait*, **6**, *8–12.*

EBBINGHAUS, HEINR.
1902. Eine neue Method zur Färbung von Hornsubstanzen. *Centbl. f. Allgem. Path. u. Path. Anat.*, **13**, *422–425.* (Methyl orange for keratin.)

EHRLICH, P.
1910. Enzyklopädie der Mikroskopischen Technik. *Second Edition. Urban & Schwartzenberg, Berlin. Vol.* **1**, *800, pp.*; **2**, *680, pp.*

EHRLICH, P., and LAZARUS, A.
1898. Die Anaemie. 1 Abt. *In Nothnagel's Spec. Path. u. Ther., Bd.* **8**, Vienna. (Describes various "neutral" stain mixtures; the "triacid" mixture; also pyronin and narcein with methyl green or methylene blue; and narcein with acid fuchsin and methyl green.)

EHRENBERG, C. G.
1838. Die Infusionsthierchen als volkommene Organismen. Leopold Voss, Leipsig. 548 pp.

ENDO, S.
1904. Ueber ein Verhaften zum Nachweiss der Typhusbacillen. *Centbl. f. Bakt., I Abt., Orig.*, **35**, *109–110.* (Proposes the fuchsin agar known as "Endo medium.")

FARIS, H. A.
1924. Neutral red and Janus green as histological stains. *Anat. Rec.*, **27**, *241–244.*

FEULGEN, R., and ROSSENBECK, H.
1924. Mikroskopisch-chemischer Nachweis einer Nucleinsäure von Typus der Thymonucleinsäure und auf die darauf beruhende elektive Färbung von Zellkernen in mikroskopischen Präparaten. *Zts. Physiol. Chem.*, **135**, *203–248.*

FEULGEN, R., and VOIT, K.
1924. Über den Mechanismus der Nuclealfärbung. *Zts. Physiol. Chem.*, **135**, *249–252;* **136**, *57–61.*
1924. Über einen weitverbreiteten festen Aldehyd. *Arch Ges. Physiol.*, **206**, *389–410.* (Use of decolorized basic fuchsin for demonstrating aldehyde in cytoplasm (plasmal reaction) by appearance of violet color.)

FISCHEL, ALFRED.
1901. Untersuchungen über Vitale Färbung. *Anat. Hefte. I Abt. Bd.* **16**, *No.* 3/4 (*Hfte. 52/3*) *417–519.* (Auramin for salamander larvae.)

FLEMMING, W.
1881. Ueber das E. Hermann'sche Kernfärbungs verfahren. *Arch. Mikr. Anat.*, **19**, *317–330.* (Magdala red as a nuclear stain. Investigated principle of differentiation with alcohol.)
1884. Mittheilungen zur Färbetechnik. *Zts. Wis. Mikr.*, **1**, *349–361.*
1891. Ueber Theilung und Kernformen bei Leukocyten, und über denen Attractionssphären. *Arch. Mik. Anat.*, **37**, *249–298.* (Triple staining technic—gentian violet, safranin and orange G—described on p. 296.)

FOOT, KATHERINE and STROBELL, ELLA CHURCH.
1905. Prophases and metaphases of the first maturation spindle of *Allolobophora foetida. Am. J. Anat.*, **4**, *199–243.* (Use of Bismarck brown for staining chromosomes in smear preparations of eggs.)

FRENCH, R. W.
1926 a. Standardization of biological stains as a problem of Medical Department of the Army. *Stain Techn.*, **1**, *11–16.*
1926 b. Azure C tissue stain. *Stain Techn.*, **1**, *79.* Fat stains. *Id.*
1926 c. Basic fuchsin for the Endo medium. *J. Lab. & Clin. Med.*, **11**, *571.*

FREY.
1868. Die Hämatoxylin färbung. *Arch. Mikr. Anat.*, **4**, *345–6.*

FROST, W. D.
1916. Comparison of a rapid method of counting bacteria in milk with the standard plate method. *J. Inf. Dis.*, **19**, *273–287.* (Use of thionin for staining young bacterial colonies.)

GERLACH, J.
1858. Mikroskopische. Studien aus dem Gebiet der menschlichen Morphologie. *Erlangen, 1858. 72 pp.* (Shows the advantage of dilute carmin solutions.)

GIEMSA, G.
1902. Färbemethoden für Malariaparasiten. *Centbl. f. Bakt., I Abt.*, 32, *307–313.* (Describes use of azur I and azur II.)
1902. Färbemethoden für Malaria parasiten. *Centbl. f. Bakt., I Abt.*, 31, *429–430.* (Showing the value of preparing blood stains with eosin and azur I alone.)
1904. Eine Vereinfachung und Vervollkommnung meiner Methylen-azur-Methelen-blau-eosin Färbemethode zur Erziehung der Romanowsky-Nochtschen Chromatinfärbung. *Centbl. f. Bakt., I Abt.*, 37, *308–311.*

GIERKE, H.
1884, 1885. Färberei zu mikroskopischen Zwecken. *Zts. Wis. Mikr.*, 1, *62–100, 372–408, 497–557;* 2, *13–36, 164–221.* (Discussion of history of staining.)

GÖPPERT, H. R., and COHN, F.
1849. Ueber die Rotation des Zellinhaltes von *Nitella flexilis. Botan. Zeitg.*, 7, *665–673, 681–691, 697–705, 713–719.* (An early use of carmin for microscopic staining purposes.)

GRAM, C.
1884. Ueber die isolierte Färbung der Schizomyceten in Schnitt und Trochenpraparaten. *Fortschritte der Med.*, 2, *185–189.*

GRENACHER, H.
1879. Einige Notizen zur Tinctionstechnik, besondes zur Kernfärbung. *Arch. Mikr. Anat.*, 16, *463–471.* (Uses alum carmin.)

GRIESBACH, H.
1882. Ein neues Tinctionsmittel für menschliche und thierische Gewebe. *Zool. Anz.*, 5, *406–410.* (Iodine green as a nuclear stain.)
1886. Weitere Untersuchungen über Azofarbstoffe behufs Tinction menschlicher und thierischer Gewebe. *Zts. Wis. Mikr.*, 3, *358–385.* (Congo red and amaranth for staining axis cylinders.)

GUYER, M. F.
1917. Animal Micrology. *Revised Edition. 289 pp. University of Chicago Press.*

HANSEN, F. C. C.
1905. Ueber Eisenhämatein, Chromalaumhämatein, Tonerdealaunhämatein, Hämateinlösungen und einige Cochenille farblösungen. *Zts. Wis. Mikr.*, 22, *45–90.*

HARRIS, D. L.
1908. A method for the staining of Negri bodies. *J. Inf. Dis.*, 5, *566–569.* (Use of alcohol soluble eosin followed by Unna's alkaline methylene blue).

HARTIG, TH.
1854. Ueber die Functionen des Zellenkerns. *Botan. Zeitg.*, 12, *574–584.* (Includes an investigation of the ability of various parts of plant protoplazm to take carmin.)
1854. Ueber das Verhalten des Zellkerns bei der Zellentheilung. *Botan. Zeitg.*, 12, *893–902.* (An early use of carmin.)
1858. Entwickelungsgeschichte des Pflanzenkeims, dessen stoffbildung während der Vorgänge des Reifens und des Keimens. *Leipzig, 1858, 164 pp.* (Uses carmin.)

HARRIS, R.
1926 a. Modification of the French azure C tissue stain. *Stain Techn.*, 1, *68–69.*
1926 b. Azure stains. *Stain Techn.*, 1, *107–111.*
1927. Investigations of thiazin dyes as biological stains: I. The staining properties of thionin and its derivatives as compared with their chemical formulae. *Stain Techn.*, 2, *8–16.*

1928. Fast green, a new substitute for light green SF yellowish. *Stain Techn.*, **3**, *40*.

HEIDENHAIN, R.

1885. Eine neue Verwendung des Haematoxylin. *Arch. Mikr. Anat.*, **24**, *468*.

1888. Beiträge zur Histologie und Physiologie der Dünndarmschleimhaut. *Pflug. Arch. Ges. Physiol.*, **43**, *Supple., 103 pp.*

HELD, HANS.

1895. Beiträge zur Structur der Nervenzellen und ihrer Fortsätze. *Arch. Anat. & Phys., Anat. Abt.*, **1895**, *396–414*. See p. 399. (For nervous tissue uses a double stain of erythrosin and methylene blue.)

HERMANN, E.

1875. Ueber eine neue Tinctionsmethode. *Tagbl. d. 48 Versaml. deut. Naturf. u. Aerzte, Graz 1875, 105 pp.* (Early use of alcohol differentiation to bring out nuclei.)

HICKSON, S. J.

1901. Staining with brazilin. *Qu. J. Micr. Sci., N. S.* **44**, *469–471*. (Brazilin after iron alum.)

HILL, J.

1770. The construction of timber, from its early growth: explained by the microscope, etc. *London, 1770.* (Use of carmin in studying the ascent of sap.) Original not seen; cited from Woodruff, 1926.

HOLMES, W. C.

1924. The influence of variation in concentration on the absorption spectra of dye solutions. *J. Ind & Eng. Chem.*, **16**, *35*.

1927. Stain Solubilities. Part II. *Stain Techn.* **2**, *68–70*.

1928. Stain Solubilities. Part III. *Stain Techn.* **3**, *12–13*.

1929. Stain Solubilities. Part IV. *Stain Techn.* **4**, *73–74*.

HOLMES, W. C., and FRENCH, R. W.

1926. The oxidation products of methylene blue. *Stain Techn.* **1**, *17–26*. (Contains technic of French azure C tissue stain.)

HUCKER, G. J. and CONN, H. J.

1923. Methods of Gram staining. *N. Y. Agr. Exp. Sta., Tech. Bul. 93.*

IRWIN, MARIAN.

1927. On the nature of the dye penetrating the vacuole of Valonia from solutions of methylene blue. *J. Gen. Phys.*, **10**, *927–947*.

JENNER, LOUIS.

1899. A new preparation for rapidly fixing and staining blood. *Lancet, 1899, Pt. 1, 370.*

JOHNSON, H., and STAUB, P.

1927. A proposed new food dye. *Ind. & Eng. Chem.*, **19**, *497–498*. (Discusses fast green FCF.)

KEHRMANN, F.

1906. Ueber Methylen-azur. *Ber. d. Deut. Chem. Gessell.*, **39**, II, *1403–1408*.

KLEBS, G.

1886. Ueber die Organisation der Gallerte bei einige Algen und Flagellaten. *Unter Bot. Inst. Tübingen*, **2**, *No. 2, 333–418*. (Congo red as reagent for cellulose; see p. 369.)

KRAUSE, R.

1926–7. Enzyklopädie der Mikroskopischen Technik. *Urban & Schwarzenberg, Berlin. 2444 pp. in 3 vols.*

KULTSCHITZKY, N.

1895. Zur Frage über den Bau der Milz. *Arch. Mikr. Anat.*, **46**, *673–695*. (Magdala red for elastic tissues.)

LEE, A. B.

1921. The Microtomists Vade-mecum. *Eighth Edition, edited by J. B. Gatenby. Blakistons, Philadelphia.*

LEE, A. B., and MAYER, P.

1907. Grundzüge der mikroskopischen Technik. *Third Edition. Berlin, 1907.*

LEISHMANN, W. B.
1901. A simple and rapid method of producing Romanowsky staining in malarial and other blood films. *Brit. Med. J., 1901*, Pt. 2, *757–758.* (Redissolved precipiate of Nocht stain in methyl alcohol.)

LEVINE, M.
1921. Bacteria fermenting lactose and their significance in water analysis. *Iowa Engineering Exp. Sta. Bul. 62.* (Use of eosin-methylene-blue agar; see p. 62–4.)

LIST, J. H.
1885. Zur. Färbetechnic. *Zts. Wis. Mikr.*, 2, *145–156.* (Uses eosin preceding methyl green.)

MACCALLUM, W. G.
1919. A stain for influenza bacilli in tissue. *J. Am. Med. Assn.* 72, *198.* (Basic fuchsin followed by picric acid and gentian violet).

MACNEAL, W. J.
1906. Methylene violet and methylene azur. *J. Inf. Dis.*, 3, *412–433.* (The history of blood stains and the chemistry of its ingredients.)
1922. Tetrachrome blood stain; an economical and satisfactory imitation of Leishmann's stain. *J. Am. Med. Assn*, 78, *1122.*
1925. Methylene violet and methylene azure A and B. *J. Inf. Dis.*, 36, *538–546.*

MACNEAL, W. J., and KILLIAN, J. A.
1926. Chemical studies on polychrome methylene blue. *J. Am. Chem. Soc.*, 48, *740–747.* (Method of preparation of azures A and B and of methylene violet from methylene blue.)
1926. Methylene azure B (Tri-methyl thionin). *Proc. N. Y. Path. Soc.*, 26, *20–23.*

MALLORY, F. B.
1891. Phospho-molybdic acid haematoxylon. *Anat. Anz.*, 6, *375–376.* (Use of hematoxylin with molybdic acid.)
1897. On certain improvements in histological technic. *J. Exp. Med.*, 2, *529–533.* (Proposes formula for phosphotungstic acid hematoxylin.)
1900. A contribution to staining methods. *J. Exp. Med.*, 5, *15–20.* (Anilin blue connective tissue stain described.)
1904. Scarlet fever. Protozoan-like bodies found in four cases. *J. Med. Res.*, 10, *483–492.* (Eosin preceding methylene blue for staining, especially in pathology.)

MALLORY, F. B., and WRIGHT, J. H.
1924. Pathological Technic. *Eighth Edition. Saunders, Philadelphia.*

MANN, GUSTAV.
1894. Ueber die Behandlung der Nervenzellen für experimentell-histologische Untersuchungen. *Zts. Wis. Mikr.*, 11, *479–494.* See p. 490. (Employs methyl blue with eosin.)
1902. Physiological Histology. *Clarendon Press, Oxford.*

MASCHKE, O.
1859. Pigmentlösung als Reagenz bei Mikroskopisch physiologisch Untersuchungen. *Bot. Zeitg.*, 17, *21–27. J. f. prakt Chem. v. Erdmann u. Wether*, 76, *37.* (First use of indigo.)

MATSUURA, S.
1925. Ueber die Färbung mit Kongorot. *Fol. Anat. Jap.*, 3, *107–110* (Congo red for staining elastic tissue.)

MAURER, S., and LEWIS, D.
1922. The cellular structure and differentiation of the specific cellular elements of the pars intermedia of the hypophysis of the domestic pig. *J. Exp. Med.*, 36, *141–156.* (In staining sections of the hypophysis, employs a mixture of acid violet and acid fuchsin, also a neutral stain combination of acid violet with safranin.)

MAYER, PAUL.
1878. Die Verwendbarkeit der Cochenille in der microscopischen Technic. *Zool. Anz.*, 1, *345–6.* (Uses Cochineal with alum.)

1891. Ueber das Färben mit Hämatoxylin. *Mitt a. d. Zool. Stat. z. Neapel*, **10**, *170–186.* (Hemalum, hemacalcum, etc.)
1892. Ueber das Färben mit Carmin, Cochenille und Hämatein Thonerde. *Mitt. a. d. Zool. Stat. z. Neapel*, 10, *480–504.*
1896. Ueber Schleimfärbung. *Mitt. a. d. Zool. Stat. z. Neapel*, **12**, *303–330.* (Describes muci-carmin, muc-hematin and gluchematin.)
1899. Ueber Hämatoxylin, Carmin, and verwandte Materien. *Zts. Wis. Mikr.*, **16**, *196–220.*

McCLUNG, C. E.
1923. Haematoxylin. *Sci.*, **58**, *515.*

MICHAELIS, L.
1900. Die vitale Färbung, eine Darstellungsmethode der Zellgranula. *Arch. Mikr. Anat.*, *55*, *558.* (Uses Janus green for chondriosomes.)
1901. Ueber Fett Farbstoffe. *Virchow's Arch. f. Path. Anat. u. Phys.*, **164**, *263.* (Proposes sudan IV.)

MÜLLER, H. A. C.
1912. Kernstudien an Pflanzen. *Arch. f. Zellforschung.* **8**, *Hft. 1, 1–51.* (Applies to plant pathology the stain mixture of Pianese—malachite green, acid fuchsin and martius yellow.)

NOCHT.
1898. Zur Färbung der Malariaparasiten. *Centbl. f. Bakt. I Abt.*, **24**, *839–843.* (First to polychromize methylene blue intentionally in preparing blood stains.)

ORTH, J.
1883. Notizen zur Färbetechnik. *Berl. Klin. Wochnsch.*, 20, *421–422.* (Proposes lithium carmin.)

OSBORNE, S. G.
1857. Vegetable cell structure and its formation as seen in the early stages of the growth of the wheat plant. *Trans. Micro. Soc.*, **5**, *104–122.* (Observes coloring of cell contents in plants grown in colored solutions—carmin, indigo, or vermilion.)

PALADINO, GIOVANNI.
1895. Della nessuna partecipazione dell' epitelio della mucosa uterina e della relative glandole alla formazione della decidua vera e riflessa nella donna. *Rend. d. Accad. Sci. fisiche e matemat. Napoli*, **34**, *p. 208–215.* (Biebrich scarlet with alum hematoxylin.)

PAPPENHEIM, A.
1898. Befund von Smegmabacillen im menschlichen Lungenswurf. *Berl. Klin. Wochnsch.*, **37**, *809.* (Rosolic acid with methylene blue in the decolorizing solution following carbol-fuchsin in staining the tubercle organism.)
1899. Vergleichende Untersuchungen über die elementare Zuzammensetzung des rothen Knochenmarkes einige Säugethiere. *Virchow. Arch. f. Path. Anat. u. Phys.*, **157**, *19–76.* (Uses mixture of methyl green and pyronin.)

PELAGETTI, M.
1904. Ueber einige neue Färbungsmethoden mit Anwendung der Zenkerschen Fixierungsflüssigkeit in der histologischen Technik der Haut. *Monatsch. f. Prak. Dermat.*, **38**, *532–536.* (Biebrich scarlet after polychrome methylene blue or after Unna's hematein. Rose bengal following hematoxylin.)

PETER KARL.
1899. Die Bedeutung der Nährzelle im Hoden. *Arch. Mikr., Anat.*, **53**, *180–211.* (Light green with hematoxylin.)

PFITZER, E.
1883. Ueber ein Härtung und Färbung vereiniges Verfahren für die untersuchung des plasmatischen zelleibs. *Ber. Deut. Bot. Gesel.*, **1**, *44–47.* (Picro-nigrosin for chromatin.)

PHILLIPS, M., and COHEN, B.
1927. The preparation of vital neutral red. *Stain Techn.* **2**, *74–79.*

PIANESE, G.
1896. Beitrag zur Histologie und Aetiologie des Carcinoms. *Beitrag zur Path. Anat. u. Allgem. Path., Suppl. I, 193 pp.* (Malachite green and martius yellow with acid fuchsin.)

PRENANT, A.
1902. Contribution a l'etude de la ciliation. *Arch. d' Anat. Micr.*, 5. (Light green with hematoxylin.)

PROESCHER, F.
1927. Oil red O pyridin, a rapid fat stain. *Stain Tech.*, **2**, *60–61.*

PROESCHER F., and ARKUSH, A. S.
1928. Metallic lakes of the oxazines (gallimin blue, gallocyanin, and coelestin blue) as nuclear stain substitutes for hematoxylin. *Stain Techn.*, **3**, *28–38.*

PRUDDEN, J. M.
1885. (A note without title). *Zts. Wis. Mikr.*, **2**, *288.* (In answer to a question by Flemming, gives formula of Delafield's hematoxylin).

RAKIETEN, M. L., and RETTGER, L. F.
1927. Brilliant green and its use in an enrichment medium in the isolation of typhoid and paratyphoid organisms. *J. Inf. Dis.*, **41**, *93.*

RANVIER.
1868. Technique microscopique. *Arch. de. Phys.* **1**, *No.* **2**, *319–321;* No. **5**, *666–670.* (First use of picro-carmin in a single procedure.)

RAWITZ, B.
1899. Bemerkungen über Karminsäure und Hämatein. *Anat. Anz.*, **15**, *437–444.*

REUTER, KARL.
1901. Uber den färbenden Bestandteil der Romanowsky-Nochtschen Malaria plasmodien färbung, seine Reindarstellung und praktische Verwendung. *Centbl. f. Bakt. I Abt.*, **30**, *248–256.* (Dissolves precipitate of Nocht stain in absolute alcohol plus anilin oil.)

ROBERTSON, O. H.
1917. The effects of experimental plethora on blood production. *J. Exp. Med.*, **26**, *221–237.* (Use of brilliant cresyl blue for staining reticulated blood cells.)

ROBINSON, H. C., and RETTGER, L. F.
1916. Studies on the use of brilliant green and a modified Endo's medium in the isolation of *Bacillus typhosus* from feces. *J. Med. Res. N. S.*, **29**, *363–376.*

ROMANOVSKI, D. L.
1891. On the question of parasitology and therapy of malaria (In Russian). *Imp. Med. Military Acad., Dissert. No. 38, St. Petersburg, 1891.* (Proposes combination of eosin and methylene blue for staining blood.)
1891. Zur Frage der Parisitologie und Therapie der Malaria. *St. Petersb. Med. Wochenschr.*, **16**, *297–302, 307–315.* A slightly condensed version of the above.

ROTHBERGER, C. J.
1898. Differential diagnostische untersuchungen mit gefärbten Nährböden. *Centbl. f. Bakt. I Abt.*, **24**, *513–518.* (Neutral red as indicator in media for differentiating typhoid and colon bacilli.)

SCALES, F. M.
1922. A new method for differential staining of bacteria. *J. Inf. Dis.*, **31**, *494–498.*

SCANLAN, J. T., FRENCH, R. W., and HOLMES, W. C.
1927. Acid fuchsin as a stain—a refinement in manufacture. *Stain Techn.*, **2**, *50–55.*

SCHAFFER.
1888. Die Färberei zum Studium der Knochenentwicklung. *Zts. Wis. Mikr.*, **5**, *1–19*. (Fast yellow for bone; congo red for embryo sections.)
SCHULTZ, G.
1923. Farbstofftabellen. *6 Aufl. Berlin. Bd. 1, 385 pp; Bd. 2, 290 pp.*
SCHWARTZ, E.
1867. Ueber eine Methode doppelter Färbung Mikroskopischer Objecte und ihre Anwendung, etc. *Sitz. berichte d. k. Acad. d. Wiss. Wien Bd.* **55**, *Hft. 1, 671–689*. (First double staining.)
SCHWEIGER-SEIDEL, F., and DOGIEL, J.
1866. Ueber die peritoneal Hülle bei Fröschen und ihren zusammenhang mit dem Lymphagefässysteme. *Ber. d. k. Sachs. Gessel. d. Wiss. zu Leipzig*, **18**, *247–254*. (Use of carminates with acetic acid.)
SCOTT, R. E., and FRENCH, R. W.
1924 a. Standardization of Biological Stains. *Mil. Surg., Aug. 1924, 15 pp.*
1924 b. Standardization of Biological Stains. II. Methylen blue. *Mil. Surg., Sept. 1924, 16 pp.*
1924 c. Standardization of Biological Stains. III. Eosin and haematoxylin. *Mil. Surg., Nov. 1924, 8 pp.*
SMITH, G. M.
1915. The development of botanical microtechnique. *Trans. Am. Micro. Soc.*, **34**, *71–129*. (Includes discussion of early history of staining.)
SMITH, J. LORRAIN.
1907. On the simultaneous staining of netural fat and fatty acid by oxazine dyes. *J. Path. & Bact.*, **12**, *1–4*. (Shows the possibility of differentiating fat and fatty acids in tissue by means of nile blue sulfate.)
SMITH, J. LORRAIN, and MAIR, W.
1911. Fats and lipoids in relation to methods of staining. *Skand. Arch. f. Physiol.*, **25**, *245–255*. (Describes the nile blue sulfate technic for staining fat in sections, as well as other methods for fat-staining.)
SMITH, LOUISE.
1920. The hypobranchial apparatus of *Spelerpes bislineatus*. *J. Morph.*, **33**, *527–583*. (Use of methylene blue in staining cartilage by the Van Wijhe technic.)
SOCIETY OF DYERS AND COLOURISTS.
1924. Colour Index. Edited by F. M. Rowe. *Published by the Society, Bradford, Yorkshire, England.*
SOEP, LEO.
1927. Le vert Janus en face du bleu de méthylène dans l'essai a la réductase selon Barthel. *Le Lait*, **7**, *927–935*. (Janus green as substitute for methylene blue in reductase test of milk.)
SPIRIDONOVITCH, R.
1924. Vital staining of white blood cells with cresylecht violet. *Anat. Rec.*, **27**, *367–373*.
SPULER, A.
1901. Ueber eine neue stückfärbemethode. *Deut. Med. Wchnschr.*, **27**, *Vereine-Beilage No.* **14**, *116*. (Iron alum cochineal.)
STILLING, H.
1886. Fragmente zur Pathologie der Milz. *Virchows Arch. f. Path. Anat. u. Physiol.* **103**, *15–38*. (Iodine green for amyloid.)
STITT, E. R.
1923. Practical Bacteriology, Blood Work, and Animal Parasitology. *Seventh Edition. Blakistons, Philadelphia.*
SUTTER, M.
1919. On the behavior of the mammary epithelial cell toward vital dyes in various functional epochs of its life cycle. *Anat. Rec.*, **16**, *164–165* (abstract). (Use of dianil blue 2 R as vital stain.)

TEICHMÜLLER, W.
1899. Die eosinophile Bronchitis. *Deut. Arch. Klin. Med.*, **63**, *444–456.* (Eosin for staining sputum, followed by methylene blue.)

TERRY, B. T.
1928. A new and rapid method of examining tissue microscopically for malignancy. *J. Lab. & Clin. Med.*, **13**, *No. 6.*

TOLSTOOUHOV, A. V.
1926. Some practical applications of the physico-chemical theory of differential staining: Blood, tissue, and bacteria staining. Methylene blue eosin water-soluble mixtures as a universal dye mixture. *Proc. N. Y. Path. Soc.*, **26**, *147–159.*

TORREY, J. C.
1913. Brilliant green broth as a specific enrichment medium for the paratyphoid-interiditis group of bacteria. *J. Inf. Dis.*, **13**, *263–72.*

TSVETT.
1911. Sur un nouveau réactif colorant de la callose. *C. R. Acad. Sci.*, **153**, *503–505.* (Use of resorcin blue—or lacmoid—as a micro-chemical reagent for callose in vegetable tissue).

TWORT, F. W.
1924 a. An improved neutral red light green double stain for staining animal parasites, microorganisms and tissues. *J. State Med.*, **32**, *351–355.*
1924 b. Further modifications in the preparation of neutral red, light-green, double stain, and an improved method of embedding tissues in paraffin. *Brit. J. Exp. Path.*, **5**, *350–351.*

UNNA, P. G.
1891. Ueber die Reifung unserer Farbstoffe. *Zts. Wis. Mikr.*, **8**, *475–487.* (Polychrome methylene blue.)
1921. Chromolyse. *Abderhalden's Handb. der Biol. Arbeitsmethoden. Abt. 5, Teil 2, Hft. 1, (Lief'ng. 17) 1–62.*

VAUGHAN, R. E.
1914. A method for the differential staining of fungus and host cells. *Ann. Mis. Bot. Gard.* **1**, *241–242.* (An adaption for botanical purposes of Pianese's stain: malachite green, acid fuchsin and martius yellow.)

VINASSA, E.
1891. Beiträge zur pharmakognostischen Mikroskopie. *Zts. Wis. Mikr.*, **8**. (Auramin for plant sections.)

WALDEYER.
1863. Untersuchungen über den Ursprung und den Verlauf des Axsencylinders bei Wirbellosen und Wirbelthieren sowie über dessen Endverhalten in der quergestreiften Muskelfaser. *Henle & Pfeifer's Zts. f. rationelle. Med., 3 Reihe, Bd.* **20**, *193–256.* (First attempt to stain with logwood extract. Early use of anilin dyes.)

WEIGERT, CARL.
1881. Zur Technik der mikroskopischen Bakterien untersuchungen. *Virchow's Arch. f. Path. Anat. u. Phys.*, **84**, *275–315.* (Gentian violet for fibrin and neuroglia.)
1884. Ausführliche Beschreibung der in No. 2 dieser Zeitschrift erwähnten Färbungsmethode für das Centralnervensystem. *Ertsch. d. Med.*, **2**, *190–191.* (Chrom-hematoxylin for medullary sheaths.)
1898. Ueber eine Methode zur Färbung elastischer Fasern. *Centbl. f. Allgem. Path. Anat.*, **9**, *289–292.* (Fuchsin for elastic tissue.)

WEIMER, B. R.
1927. The use of nile blue sulfate as a vital stain on Hydra. *Biol. Bul.*, **52**, *219–222.*

WILLIAMS, B. G. R.
1923. Cresylecht violet, a rare dye. *J. Lab. & Clin. Med.*, **8**, *No. 4, Jan. 1923. 4 pp.*

1925. Further studies with cresylecht violet, including a report of my six-second method for staining tissue. *J. Lab. & Clin. Med.*, **10**, *312–315.*

WINOGRADSKY.

1924. Sur l'etude microscopique du sol. *C. R. Acad. d. Sci.* **179**, *367.* (Erythrosin for staining bacteria in soil.)

WOODRUFF, L. L.

1926. The versatile Sir John Hill, M.D. *Amer. Nat.*, **60**, *417–442.*

ZIMMERMANN, A.

1893. Beiträge zur Morphologie und Physiologie der Pflanzenzelle. Bd. II. *Tübingen*, 1893. *35 pp.* (Iodine green as chromatin stain for plant cells.)

ZSCOKKE, E.

1888. Ueber einige neue Farbstoffe bezüglich ihrer Verwendung zu histologischen Zwecken. *Zts. Wis. Mikr.*, **5**, *465–470.* (Benzopurpurin in contrast to hematoxylin.)

REPORTS OF COMMISSION ON STANDARDIZATION OF BIOLOGICAL STAINS AND OF RELATED COMMITTEES

COM. ON BACT. TECHNIC, OF SOC. AMER. BACTERIOLOGISTS, H. J. Conn, chairman.

1921. The Production of Biological Stains in America. *Science*, **53**, *289–290*.

1922a. An Investigation of American Stains. *J. Bact.*, **7**, *127–148*.

1922b. An Investigation of American Gentian Violets. *J. Bact.*, **7**, *529–536*.

COM. ON STANDARDIZATION OF BIOL. STAINS, OF NAT. RES. COUNCIL, H. J. Conn, chairman.

1922a. The Standardization of Biological Stains, *Science*, **55**, *43–44*.

1922b. American Biological Stains compared with those of Grübler. *Science*, **55**, *284–285*.

1922c. Preliminary Report on American Biological Stains. *Science*, **56**, *156–160*.

1922d. Collaborators in the Standardization of Biological Stains. *Science*, **56**, *594–596*.

COMMISSION ON STANDARDIZATION OF BIOL. STAINS, H. J. Conn, Chairman.

1922a. The Present Supply of Biological Stains. *Science*, **56**, *562–563*.

1922b. American Eosins. *Science*, **56**, *689–690*.

1923b. The preparation of Staining Solutions. *Science*, **57**, *15–16*.

1923c. Safranin and Methyl Green. *Science*, **57**, *304–305*.

1923d. Thionin. *J. Dairy Science*, **6**, *135–136*.

1923e. Dye Solubility in Relation to Staining Solutions. *Science*, **57**, *638–639*.

1923f. Standardized Nomenclatures of Biological Stains. *Science*, **57**, *638–639*.

1923g. Certified Methylene Blue. *Science*, **58**, *41–42*.

1924a. Investigations Concerning Imported Biological Stains. *Science*, **54**, *328–331*.

1924b. Certified Safranin. *Science*, **54**, *556–557*.

1924c. A report on Basic Fuchsin. *Science*, **60**, *378–388*.

1925a. Certified Stains—What They Are and How to Obtain Them. *J. Lab. and Clin. Med.*, **10**, *321–322*.

1925b. New Applications of Biological Stains. *J. Chem. Education*, **2**, *184–185*.

1927 Commission Specifications of Certain Stains. *Stain Techn.* **2**, *27–30*.

INDEX

In this index the dyes named are printed either in bold-faced type or italics; preferred designations are in bold-faced type, synonyms in italics. Figures in bold-faced type indicate the principal references.